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LABORATORY TECHNIQUES IN RABIES

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IN

RABIES

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CONTENTS

Page

PART III. VACCINE POTENCY TESTS (*continued*)

PART IV. PRODUCTION OF HYPERIMMUNE SERUM

PART V. LABORATORY ANIMALS

ADDITIONAL REFERENCE SOURCES

FOREWORD

The World Health Organization has in the past received many requests for information on laboratory techniques connected with various aspects of rabies. As a result of the need for this kind of information in countries of the Eastern Mediterranean, South-East Asia, and Western Pacific Regions of WHO, a meeting was organized for these countries in July 1952 at the Pasteur Institute of Southern India in Coonoor. The meeting provided for lectures, discussions, demonstrations, and laboratory training, and was attended by 55 medical and veterinary officials, including 7 discussion leaders, from 23 different countries WHO consultants on rabies acted as discussion leaders and supervised the laboratory sessions. The working papers, in particular the laboratory instructions prepared by well-known authorities, proved most useful, and it was decided to expand and revise the material for publication The WHO Expert Committee on Rabies, at its second session in Rome in September 1953, discussed the projected manual in detail and made frequent references to it in their report The reports* of the committee deal in general with the overall problems encountered in rabies, including prophylaxis in human beings and control in animals, whereas this publication is concerned solely with laboratory aspects of the disease

The manual is not intended as an exhaustive treatise, its scope has been purposely limited to one or two procedures in each of the major divisions of laboratory techniques in rabies The contributors were requested to select and present procedures based on their own experience which would be dependable and practicable without sacrificing necessary minimal standards, but which at the same time could be adapted to the limited facilities and personnel of many rabies laboratories in different parts of the world The techniques were selected also with a view to encouraging and facilitating uniform methods which would permit of a more valid comparison of results obtained in different laboratories

Certain features of the various sections require some explanation.

* *Wld Hlth Org techn Rep Ser* 1950, 28 1953 82

Detection of Negri bodies by rapid techniques is the aim of every diagnostic laboratory dealing with rabies, and a choice must be made from a multiplicity of methods described by different authorities. It is believed that the impression method presented here can be mastered relatively easily by most laboratory technicians. Where histopathological sections are concerned, more-specialized training in pathology is required and this part of the manual has been arranged for such trained individuals.

In the laboratory diagnosis of rabies, examination is frequently limited to the tissues of the central nervous system, and the salivary glands are entirely overlooked. It has been aptly stated that "animals do not bite with their brains"—the real risk of contracting rabies is dependent on whether or not virus is present in the saliva of a biting animal. However, salivary excretion of virus does not occur in an appreciable but unpredictable number of cases where virus can be recovered or detected in the brain of the animal. Examination of the submaxillary salivary gland for the presence of virus by mouse inoculation should therefore be carried out wherever possible concurrently with nervous-tissue examination.

The necessity for mouse inoculation tests in routine diagnosis is worthy of special emphasis. Careful studies have shown that up to 20% of animals negative on examination for Negri bodies were positive for rabies by the mouse inoculation test.

A description of the preparation of the Semple phenolized type of vaccine, as representative of the most widely used of the "killed" vaccines, is given. This does not imply that other inactivated or living virus vaccines such as the Hempt-, Högyes-, Fermi-, or Harris-type vaccines, to name a few, are not equally effective, provided they are adequately tested for potency Two methods for producing the Semple-type vaccine are given: one according to the procedure used at the Institut Pasteur, Paris, and the other to meet the requirements of the National Institutes of Health of the United States of America These methods were chosen because they cover the largest number of requests for information received by WHO. It is recognized that modifications of these methods are used successfully in many countries.

Descriptions of the production of ultraviolet-light inactivated vaccine and modified virus vaccine prepared from chicken embryos are included because of wide interest in these effective and relatively new products. Commercial or large-scale preparation of potent rabies vaccines from which the paralysis-producing factor has been removed has not, as yet,

been successfully accomplished, and this procedure is therefore omitted from the manual.

The necessity for performing potency tests on each batch of vaccine cannot be too strongly emphasized, for ample experience has shown that even when routine procedures are closely followed in the preparation of successive batches of vaccine there is no automatic assurance of a potent product. Potency tests in laboratory animals provide our only basis, at present, for any degree of certainty that a vaccine possesses sufficient immunogenicity to give dependable results in human beings or animals. Several potency tests of varying complexity are given so that a laboratory may select, or devise, one most suitable to its local conditions and facilities. In the latter instance, strongest consideration should be given to vaccination followed by challenge with street virus, always with an adequate number of control animals. For this purpose dogs are suitable and can be considered as the animal of choice. The important factor to be demonstrated in control tests of rabies vaccines is immunogenicity and not merely innocuity, a mistake which is not uncommon.

Hyperimmune serum is a promising addition to rabies prophylaxis in man and animals; Part IV is devoted to a description of its preparation, and of potency tests. Laboratory investigations on hyperimmune serum now under way, co-ordinated by WHO, should give us more information within the next year or two on its prophylactic value. The WHO Expert Committee on Rabies dealt with this subject at its second session (see footnote * on page 9)

Part V is devoted to some of the more important problems associated with the use in rabies work of small laboratory animals, a frequent source of difficulty in many countries. The section is necessarily brief and touches on only the chief aspects of the subject—in particular, diseases encountered in these animals which might affect experimental results. Further information on this topic, and on material dealt with elsewhere in the manual, may be obtained from the additional reference sources given on page 147

Work with viruses is a highly developed discipline which permits of little latitude if reproducible results are to be obtained. However, it is to be expected that the opinion of individual workers on techniques will differ with respect to details The techniques recommended here have been prepared for particular application in rabies work, although it is evident that some of them, such as the serum-virus neutralization test and the mouse inoculation test, are readily applicable, perhaps with slight

modifications, to other virus diseases. It will be noted further that in describing the various techniques a rational and systematic approach has been stressed by the contributors so that errors which might otherwise nullify excellent work may be avoided. An example which may be cited is the advisability of challenging vaccinated animals before, or alternately with, control animals in determining the potency or effectiveness of a vaccine.

Rabies research is far from static, and it is to be expected that modifications of some of the procedures described will be evolved in the rather near future. It is felt, however, that the techniques given in this manual should be suitable for several years to come, as they are the result of extensive and proven experience.

* * *

WHO is grateful to the contributors for the care and trouble they have taken in this work, much of which was hitherto either unpublished, or not readily available in compact form in scientific literature Many of the contributors to this manual are members of the WHO Expert Advisory Panel on Rabies, and their contributions record only a part of their valuable services to the Organization in striving for greater successes in the control of this dreaded disease

frozen brain should remain at room temperature until thawed before
ection

The following information is desirable when animal heads are received
examination the species and breed of the animal, and whether it
in contact with other animals, whether the animal died or was killed,
if the latter, the means used for destroying it, whether the animal was
ned and observed for an appropriate time before death, and, if so,
ow long, symptoms of rabies, if any, history of vaccination against

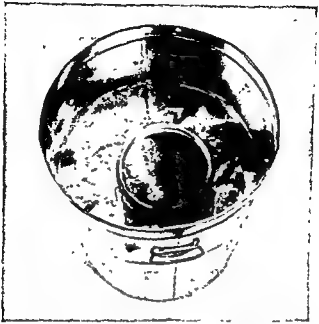
Removal of Animal Brain

precautions, including careful operative technique and protection
hands with heavy rubber autopsy-gloves, should be taken against
ssibility of infection of persons opening animal heads

head should be held firmly on a solid table, proper immobilization
effected by grasping it with a lion-jaw type of bone-holding forceps

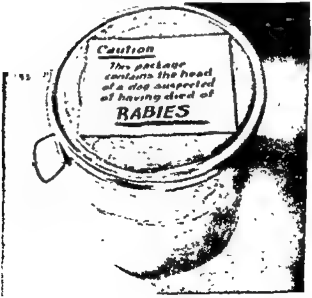
IG 3 INITIAL SKIN INCISION ALONG MIDLINE OF DOG'S HEAD

FIG. 1. DOUBLE CONTAINER WITH ICE FOR PACKING SPECIMENS



By courtesy of United States Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

FIG. 2. CONTAINER COVERED AND LABELLED



By courtesy of US DHEW-PHS-CDC

the frozen brain should remain at room temperature until thawed before dissection

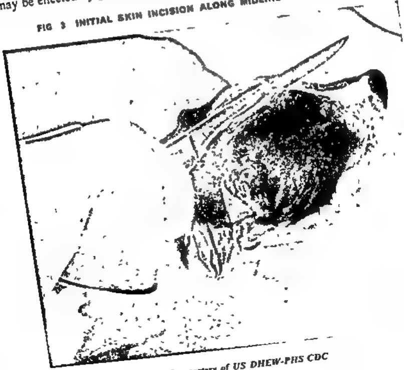
The following information is desirable when animal heads are received for examination: the species and breed of the animal, and whether it was in contact with other animals; whether the animal died or was killed, and, if the latter, the means used for destroying it; whether the animal was confined and observed for an appropriate time before death, and, if so, for how long; symptoms of rabies, if any; history of vaccination against rabies.

Removal of Animal Brain

All precautions, including careful operative technique and protection of the hands with heavy rubber autopsy-gloves, should be taken against the possibility of infection of persons opening animal heads.

The head should be held firmly on a solid table, proper immobilization may be effected by grasping it with a lion-jaw type of bone-holding forceps

FIG 3 INITIAL SKIN INCISION ALONG MIDLINE OF DOG'S HEAD



By courtesy of US DHEW-PHS CDC

FIG. 4. USE OF BONE-SAW FOR MAKING TRANSVERSE ANTERIOR INCISION IN FRONTAL BONES JUST ABOVE THE EYES

By courtesy of US DHEW-PHS-CDC

FIG. 5. CALVARIA REFLECTED

By courtesy of US DHEW-PHS-CDC

firmly applied to the maxilla. Improvised mechanical devices such as a carpenter's vice have often also been found effective.

With section knife or large scalpel a midline incision is made through the skin, fasciae, and muscles of the cranium, beginning anteriorly just above the level of the eyes and extending posteriorly to the base of the skull (see fig 3) The skin, fasciae, and temporal muscles are then dissected away from the cranium and reflected laterally exposing the bone The calvaria is then removed by means of a saw, bone-chisel, or butcher's cleaver. The sawing method is preferred by most laboratories. In this procedure a sterile surgical bone-saw is used to incise the bone of the skull on each side, beginning at the foramen magnum and then sawing anteriorly to the frontal bones The longitudinal cuts are then joined by a transverse incision through the frontal plate just above the eyes (see fig 4) The calvaria is then lifted off with the aid of bone-cutting forceps and/or bone-chisel (see fig 5)

With a fresh set of sterile instruments, the brain is now removed from the cranium. The meninges and the tentorium cerebelli which separates the cerebrum and cerebellum are dissected away with rat-tooth thumb forceps and either scalpel or sharp-pointed scissors. Next, with scalpel or scissors reaching back into the posterior portion of the brain, the brain is severed from its site by cutting into the medulla, cranial nerves, and anterior extension of the thalamus The entire brain is then lifted out of the cranium onto a paper picnic-plate or a large Petri dish (see fig 6)

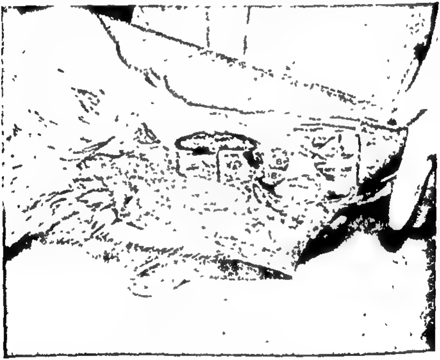
FIG. 6 REMOVAL OF ENTIRE BRAIN

By courtesy of US DHEW-PHS-CDC

If the body of the animal is available and no typical Negri bodies are found in the rapidly stained films, make a general post-mortem examination in an attempt to determine the reason for the illness or unusual behaviour of the animal. Wrap the remains in paper and ensure their incineration. Wash the table with 10% cresol solution. Sponge the gloves in the same kind of solution before removing them from the hands, and then sterilize them, with the instruments, by boiling.

It is always advisable to record the gross appearance of the brain, indicating especially the state of preservation, the presence or absence of

FIG. 7. SKIN REFLECTED AND SUBCUTANEOUS TISSUE REMOVED, EXPOSING SUBMAXILLARY SALIVARY GLAND



A Submaxillary salivary gland
B Submaxillary lymph glands

adhesions and exudate, and whether the blood-vessels are injected. There are no gross pathological changes which can be regarded as diagnostic of rabies.

Even in the field, precautions should be taken to prevent bacterial contamination of the animal brain by the use of sterile instruments in brain removal and dissection

Removal of Salivary Glands

The examination of the salivary glands for the presence of virus is of obvious value in providing definitive evidence of whether or not a bite has entailed a risk. Presence of the infection in the central nervous system does not necessarily indicate infective saliva.

If virus is present in the salivary glands, the submaxillary (mandibular) glands will contain the most virus For removal of the submaxillary salivary glands, the head is turned over so that the ventral aspect is facing upward.

FIG 8 LEFT SUBMAXILLARY SALIVARY GLAND COMPLETELY EXCISED

A Submaxillary salivary gland
B Submaxillary lymph glands

With autopsy knife or scalpel, a midline incision is made in the skin covering the area between the rami of the mandible The incision should begin at the mandibular lip and extend posteriorly to the neck The skin is then reflected laterally, exposing the muscles and superficial soft tissues of the lower jaw

The submaxillary salivary gland on each side is situated quite superficially and after reflecting the skin back it can be seen in the area of the posterior border of the mandible behind and below the superficial submaxillary lymph glands, with which the salivary glands should not be confused (see fig. 7 and 8). The submaxillary salivary gland is elliptical in shape, about 5 cm long and 3 cm wide; it is rounded in outline, greyish-yellow or orange in colour, and covered by a fibrous capsule.

With a fresh sterile scalpel and rat-tooth thumb forceps, the gland on each side is dissected away from its surrounding tissues and placed in a Petri dish

A small portion of each salivary gland is cut with sterile scissors and pooled in a single mortar for preparation of the salivary-gland suspension for the mouse inoculation test (see section 4, page 57) The test pieces should be weighed before emulsification for calculation of the concentration of the tissue suspension (see page 47 for gasserian ganglion)

Glycerinated Specimens

When facilities for animal inoculation of Negri-negative or doubtful animal brains suspected of rabies infection are not at hand, it is possible to ship portions of brain or salivary gland to a laboratory which performs animal inoculation tests

A solution of sterile 50% glycerol-saline is prepared by adding equal parts of chemically pure glycerol to physiological salt solution. This solution may then be placed in small bottles or jars with screw tops, autoclaved, and stored at room temperature.

Select fairly large portions of brain or salivary gland, at least about the size of a mouse brain (0.3 g or more) in order to give the laboratory worker sufficient material with which to work. Portions of the brain should include parts of the hippocampus, cerebellum, and cerebral cortex from each side. Also take a portion of the medulla or brain stem. Place the tissue pieces in a bottle of prepared 50% glycerol-saline. Rabies virus, if present, will be preserved during shipment, and no refrigeration is required

Glycerinated portions of brain do not usually produce satisfactory smears on a slide since it is difficult to make the glycerinated brain adhere to a slide. Furthermore, the staining properties of such tissues are changed, making recognition of inclusion bodies much more difficult. For this reason, if further examination of brain smears on the questionable specimen is desired, smears may first be made from the unpreserved brain and stained, and the slides included in shipment along with the glycerinated brain.

Alternatively, brain smears may be made and the *unstained* slides plunged immediately into chemically pure methyl alcohol (acetone-free) for fixation, and then removed and dried at room temperature without blotting. These unstained, fixed smears can then be stained at the laboratory to which they are referred.

Upon their receipt in the laboratory, glycerinated tissue specimens should be immediately removed from the shipping bottle and placed in a sterile Petri dish. The pieces of tissue are then thoroughly washed in physiological saline solution by continual addition of the solution, gentle rotary agitation of the Petri dish, and discarding of the liquid. This is repeated several times. At this point it is as well to attempt a smear preparation of the washed brain-tissue specimens on a slide for Negri-body staining and microscopic examination. The quality of the smear will depend on the amount of glycerol washed from the brain tissue. The remaining specimens are now ready for emulsification and mouse inoculation. For grinding salivary glands in a mortar a small amount of sterile sand or alundum is essential, but these materials are superfluous for making brain suspensions.

Note. It is advisable to retain in glycerol solution some unused portions of all animal-tissue specimens until the animal inoculation test either has been reported positive for rabies, or has run its course (at least 21 days), and has been reported negative for rabies.

Section 2 *

RAPID MICROSCOPICAL EXAMINATION FOR NEGRI BODIES USING SIMPLE TISSUE-APPLICATION TECHNIQUES, AND PREPARATION OF SPECIMENS FOR BIOLOGICAL TEST

The techniques employed in the laboratory diagnosis of rabies should embrace optimum conditions of accuracy, speed, and economy. The method employing the microscopical examination for Negri bodies, using the simple application of brain tissue to a slide and Sellers' technique for staining (see page 32), has been proved to fulfil these requirements.

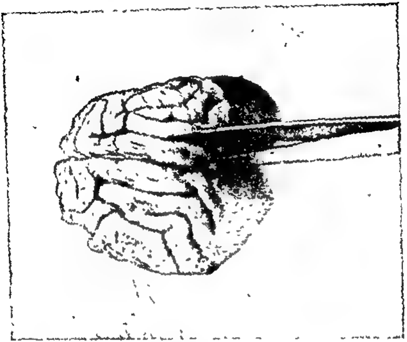
It has been found that Negri bodies, when present, are most readily demonstrated in Ammon's horn (hippocampus major) of the brain and also in the pyramidal cells of the cerebral cortex and Purkinje's cells of the cerebellum; they are found to a much more limited extent in the neurons of the thalamus, pons, medulla, spinal cord, and sensory ganglia.

Dissection of the Brain

A very simple operation is required to expose Ammon's horn, which is generally the best area for demonstration of Negri bodies in most species of rabid animals With a pair of sterile scissors, a longitudinal incision is made into the dorsal surface of each cerebral hemisphere, about 2 cm lateral to the longitudinal fissure or midline of the brain (see fig 1). The incision is made from the region of the occipital pole of the hemisphere and is extended forward for 3-5 cm and downward through the grey matter, and then completely through the white matter until a narrow space, the lateral ventricle, is reached The opening is then widened by spreading the incised hemisphere, and Ammon's horn will be revealed as a semi-cylindrical, white, glistening body bulging laterally from the ventricle floor (see fig 2 and 3) It has a spiral contour and, on cross-section, a characteristically rolled surface

* Contributed by Ernest S Tierkel, Director Rabies Control Activities Communicable Disease Center Public Health Service US Department of Health, Education and Welfare Atlanta, Ga, USA

FIG. 1. SITE OF INCISION FOR LOCATING AMMON'S HORN



By courtesy of United States Department of Health, Education and Welfare Public Health Service, Communicable Disease Center (US DHEW-PHS-CDC)

Preparation of Slide

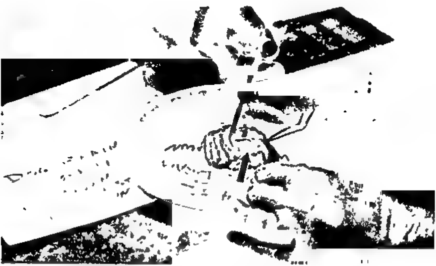
Slides should be made first from Ammon's horn, then from the cerebral cortex, and finally from the cerebellum. Samples (at least six) should be taken from these three areas on each side of the brain and examined microscopically before the brain is reported Negri-negative. It is always wise to select another area from each hippocampus for good measure.

Three recommended methods of applying fresh brain tissue to slides are as follows

Impression method

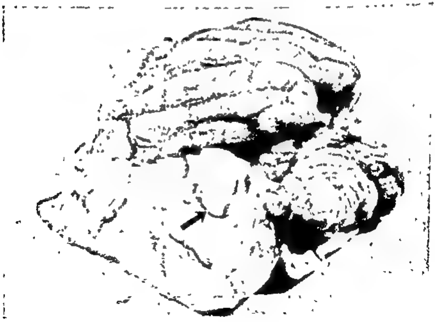
With a pair of scissors, small transverse sections (2-3 mm in thickness) of brain tissue (Ammon's horn, cerebrum, or cerebellum) are cut and placed on clean blotting-paper or a wooden tongue-depressor, cut surface

FIG. 2. INCISION TO LATERAL VENTRICLE SEPARATED, SHOWING AMMON'S HORN*



By courtesy of US DHEW-PHS-CDC

FIG. 3. CLOSE-UP OF AMMON'S HORN* BULGING FROM FLOOR OF LATERAL VENTRICLE



By courtesy of US DHEW-PHS-CDC

* Indicated by arrow

facing upward (see fig. 4 and 5) A clean micro-slide is then touched against the cut surface of the section and pressed gently downwards with just enough pressure exerted to create a slight spread of the exposed surface of the tissue against the glass slide. According to the size of the section, three to four impressions can be made on one slide (see fig. 6 and 7).

FIG. 4. TAKING OF SPECIMEN FROM AMMON'S HORN FOR SLIDE PREPARATIONS

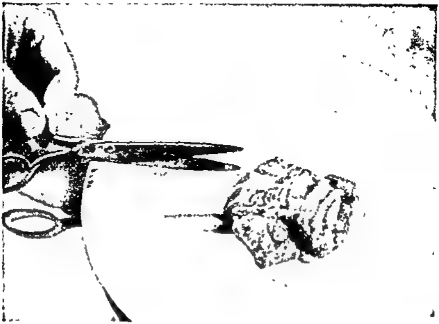
By courtesy of US DHEW-PHS CDC

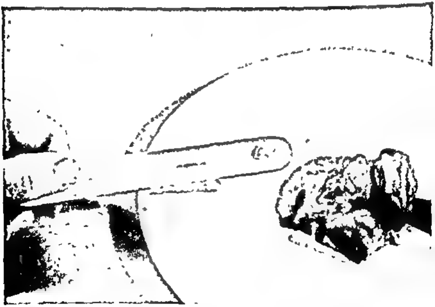
While still moist, the slide is flooded with Sellers' stain (see fig 8), allowed to remain for a few seconds, rinsed under the tap, and dried at room temperature without blotting The preparation is then ready for examination The impression may be examined directly under oil, or covered with a cover-slip mounted in balsam This method is preferred over others because a maximum amount of nerve tissue can be concentrated in a small area with a minimum amount of cellular damage

Smear method

The spread-smear method consists of placing a very small section of brain tissue on one end of the slide. Another slide is used to crush the

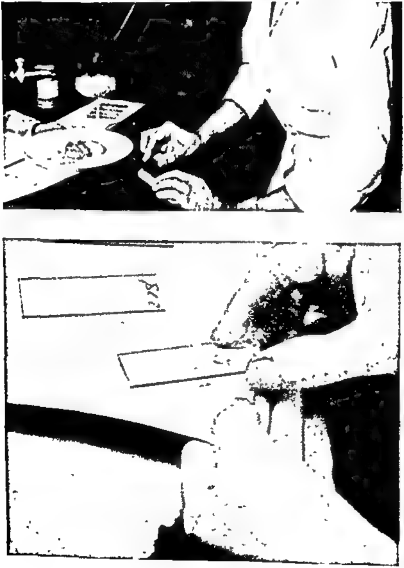
FIG. 5. TRANSFER OF SPECIMEN TO WOODEN SPATULA BEFORE MAKING IMPRESSIONS





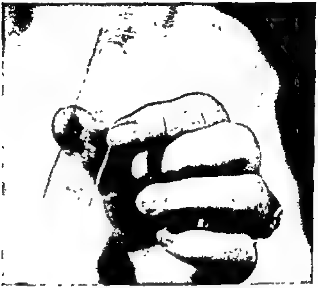
By courtesy of US DHEW-PHS-CDC

FIG. 6. IMPRESSION METHOD OF SLIDE PREPARATION



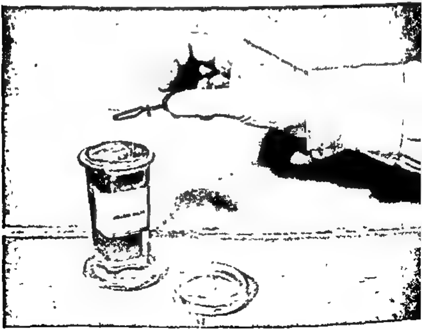
By courtesy of US DHEW-PHS-CDC

FIG. 7. CLOSE-UP OF SEVERAL IMPRESSIONS ON A SLIDE



By courtesy of US DHEW-PHS-CDC

FIG. 8 IMMEDIATE IMMERSION OF SLIDE IN SELLERS' STAIN WHILE TISSUE FILM IS STILL MOIST



By courtesy of US DHEW-PHS-CDC

section of tissue against the first slide and is then drawn across the length of the slide (see fig 9). The result is a fairly homogeneous spread of a thin film of tissue covering about three-quarters of the area of the slide.

FIG. 9. SMEAR METHOD OF SLIDE PREPARATION

By courtesy of US DHEW-PHS-CDC

In this spread-smear technique there is a copious concentration of tissue and a rather extensive area for examination. Care should be taken not to use too large a tissue-section, which will result in an excessively thick film, making proper staining and microscopic examination impossible. The impression method, however, gives superior results

"Rolling" method

The last method, the "rolling" technique, consists of cutting a piece of brain tissue about the size of a fresh garden-pea, and rolling or teasing it gently (cut surfaces downward) over the entire surface of the slide with a toothpick or wooden applicator

The staining procedure of Sellers is recommended here because of its accuracy and simplicity In this technique, no preliminary fixation is required, since the tissue film is fixed and stained simultaneously, making it one of the most rapid and easily handled methods

Preparation of Sellers' Stain *

Examination of slide

Time may be saved in microscopic examination by a study of the stained slide under low power at first, selecting areas containing numerous large neurons to be examined for Negri bodies under immersion oil (see fig 10 and 11).

FIG. 10. LOW-POWER VIEW OF IMPRESSION, SHOWING FIELD (UPPER HALF) RICH IN NEURONS FOR EXAMINATION UNDER HIGH POWER

By courtesy of US DHEW-PHS CDC

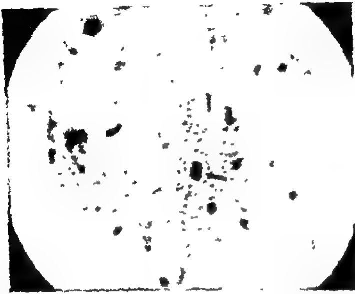
Magnification ×200

Sellers' stain shows the Negri body well-differentiated in magenta or heliotrope to bright red, with well-demonstrated dark-blue to black basophilic inner bodies. All parts of the nerve cell stain blue, and the interstitial

* This subsection was very kindly contributed by Thomas F. Sellers Director, Georgia Department of Public Health Atlanta Ga USA

tissue stains pink. Erythrocytes stain copper-colour (orange-tinged red) and can be easily differentiated from the magenta-tinged red of the Negri bodies (see colour plate, fig. 1, facing page [illegible]).

FIG. 11. HIGH-POWER VIEW OF NEGRI BODY*



By courtesy of U.S. DHEW-PHS-CDC

* Indicated by arrow

Magnification ×[illegible]

Stock solution

(1) Methylene blue (Colour Index[1,2] No. 922, or Schultz[3] Index No. [illegible]) . . . . 10 g
Methyl alcohol (absolute acetone-free) to make . . . . . 1000 ml

(2) Basic fuchsin (Colour Index No. 677, or Schultz No. 780) . . . 5 g
Methyl alcohol (absolute acetone-free) to make . . . 500 ml

Other suitable dyes, such as those from Grübler & Co., Leipzig, Gurr & Co., Ltd, London, and National Aniline Co., New York, may also be used.

[1] Society of Dyers and Colourists (1924) F. M. Rowe, ed., *Colour index*, Bradford
[2] Society of Dyers and Colourists (1928) F. M. Rowe, ed., *Supplement to colour index*, Bradford
[3] Schultz, G. (1923-1924) *Farbstofftabellen*, 7. Aufl., Leipzig

Preparation of Sellers' Stain *

Examination of slide

Time may be saved in microscopic examination by a study of the stained slide under low power at first, selecting areas containing numerous large neurons to be examined for Negri bodies under immersion oil (see fig 10 and 11).

FIG. 10. LOW-POWER VIEW OF IMPRESSION, SHOWING FIELD (UPPER HALF) RICH IN NEURONS FOR EXAMINATION UNDER HIGH POWER

By courtesy of US DHEW-PHS-CDC

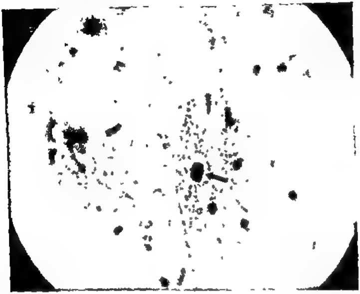
Magnification ×200

Sellers' stain shows the Negri body well-differentiated in magenta or heliotrope to bright red, with well-demonstrated dark-blue to black basophilic inner bodies. All parts of the nerve cell stain blue, and the interstitial

* This subsection was very kindly contributed by Thomas F Sellers Director, Georgia Department of Public Health Atlanta Ga USA

tissue stains pink. Erythrocytes stain copper-colour (orange-tinged red) and can be easily differentiated from the magenta-tinged red of the Negri bodies (see colour plate, fig. 1, facing page 40).

FIG 11. HIGH-POWER VIEW OF NEGRI BODY*



By courtesy of US DHEW-PHS CDC

* Indicated by arrow   Magnification ×800

Stock solution

(1) Methylene blue (Colour Index[1,2] No 922, or Schultz[3] Index No. 1038)	10 g
Methyl alcohol (absolute acetone-free) to make	1,000 ml
(2) Basic fuchsin (Colour Index No 677, or Schultz No. 780)	5 g
Methyl alcohol (absolute acetone-free) to make	500 ml

Other suitable dyes, such as those from Gruebler & Co., Leipzig, Gurd & Co , Ltd, London, and National Aniline Co., New York, may also be used

[1] Society of Dyers and Colourists (1924) F Rowe, ed , *Colour index*, Bradford

[2] Society of Dyers and Colourists (1928) F Rowe, ed , *Supplement to colour index*, Bradford

[3] Schultz, G (1928-1934) *Farbstofftabellen* 7 Aufl , Leipzig

rinsed with tap-water and others rinsed in distilled water containing M/150 phosphate buffer, pH 7.0.

The stain must be kept in a tightly-closed container when not in use, to prevent evaporation, which tends to make the fuchsin too dominant. The addition of absolute methyl alcohol will restore the proper balance. It is convenient to keep the staining solution in a screw-capped Coplin jar for daily use. If this is not available the stain may be stored in a ground-glass-stoppered dropper bottle and the smear flooded with the stain. Staining in this manner will not be satisfactory unless the entire process can be completed within a few seconds.

The best results with the stain are obtained when the brain tissue is fresh. As decomposition sets in, the characteristic colour differentiation is affected, and although the Negri bodies retain their staining quality, the smear as a whole becomes too red, or at times too blue, and identification of the bodies becomes more difficult.

The Negri Body: Differential Diagnosis

Although generally rounded in form, the Negri body may be found to assume any shape. At various times in different laboratories it has been demonstrated to be round, oval, spheroid, amoeboid, elongate, triangular, etc. By the same token, there is great variation in size; generally it is found within the limits of 0.24 μ and 27.0 μ. It is characteristically acidophilic in staining reaction, and takes on the pink to purplish-pink colour in differential stains which use basic fuchsin or eosin with methylene blue as their base.

The position of the Negri body within the neuron is intracytoplasmic. Classically, it is found between the nucleus and one corner of the neuron, or in the prolongation of the cell body. However, it should be stressed here that the intracytoplasmic position of the Negri body can be expected with reasonable consistency only in histological sections of the brain. In the simple tissue-application techniques described above, the histological pattern is disturbed and one may very often see well-formed Negri bodies which appear to be entirely outside the neuron. Thus, in methods such as the impression, smear, or rolling techniques, the intracellular position of the Negri body is not required as a diagnostic criterion, and Negri bodies which satisfy the requisites of morphological identification, whether inside or outside the neuron, are sufficient to establish a positive diagnosis.

The most characteristic feature of the Negri body is its internal structure. It is this feature which serves as the most essential criterion for positive identification in the techniques described in this section. The matrix of the Negri body has an acidophilic staining reaction, and contained within this magenta-red structure are small inner bodies (Inner-

körperchen), basophilic granules which stain dark-blue to black. The size of these inner granules generally varies from 0.2 μ to 0.5 μ. Classically, the well-formed Negri body—the so-called textbook picture—will have its inner granules arranged in rosette fashion, with one large centrally-placed body and a series of smaller granules arranged neatly around the periphery of the Negri body. It should be pointed out, however, that this picture is the exception rather than the rule, and it is very rare indeed that such an orderly arrangement of the inner granules is seen. For purposes of diagnosis it is sufficient to establish the presence of these dark-blue-staining granules, regardless of their numbers or pattern of distribution within the matrix of the Negri body.

There is universal agreement that the Negri body is specific for rabies, and its presence always indicates this infection. Furthermore, a fully-formed Negri body cannot be confused with anything else. However, in the diagnostic laboratory other types of inclusion bodies are sometimes encountered in animal brains and, because of certain similarities, may be mistaken for Negri bodies. This is particularly true of the dog, fox, cat, and laboratory white mouse. In the brains of dogs and foxes, the acidophilic inclusion bodies of canine distemper or Rubarth's disease (canine infectious hepatitis, fox encephalitis) are occasionally encountered These seem to occur more often in the thalamus and lentiform nuclei than in the hippocampus. By the same token, the brains of non-rabid cats and laboratory white mice occasionally contain non-specific acidophilic inclusion bodies when presented for rabies diagnosis. All these non-rabies *inclusion bodies have the same staining characteristics for Sellers'* stain, and they cannot be differentiated from each other with the techniques described above. However, the important thing is that these non-rabies inclusions, as a group, can be differentiated from Negri bodies with the use of Sellers' stain. The following outline may be used as a guide in this differentiation

Negri bodies	*Non-rabies inclusion bodies*
Presence of basophilic inner granules	Absence of internal structure *
Heterogeneous matrix	Homogeneous matrix
Less refractile	More highly refractile
Magenta (heliotrope) tinge	Colour more acidophilic (pinker)

* See colour plate fig 2, facing page 40

Small atypical intracytoplasmic inclusion bodies are sometimes found in animals killed during the early stages of rabies. For that reason, it is imperative to hold biting and suspect dogs in quarantine, rather than to kill them immediately and send the brain to a laboratory for diagnosis (see page 15) There is a double reason for this. First, it will allow of

observation for symptoms of rabies which may make possible a clinical diagnosis of the disease. Secondly, the longer the animal is allowed to live, the better the chance of obtaining a positive microscopic diagnosis. The length of clinical illness in rabies is directly related to the presence, size, abundance, and development of Negri bodies. Thus, if the disease runs its full course, Negri bodies which are larger, more abundant, and fully formed with good internal structure, are more likely to be found.

Biological Diagnostic Test: Preparation for Mouse Inoculation

Since Negri bodies cannot always be found in the brains of animals dying of rabies, it is important that animal inoculation for demonstration of the virus be done on Negri-negative specimens. Extensive surveys of large numbers of rabies cases have shown that 10%-15% of those cases proved positive by mouse inoculation had been missed by direct-smear microscopic examination for Negri bodies. It is therefore strongly recommended that laboratories which furnish rabies diagnostic services be equipped to carry out animal inoculation tests on Negri-negative brain tissues.

In the past the guinea-pig and rabbit have been considered the most suitable test animals for this purpose. Since the demonstration that the intracerebral injection of rabies virus into white mice produces typical and constant infection, the white mouse has become the test animal of preference. The chief advantages of the mouse are the low cost, making it possible to use several animals for one specimen, the relatively short incubation period for street virus, and the consistency of production of Negri bodies in the brains of mice inoculated intracerebrally with street virus.

A positive microscopic diagnosis is sufficient proof of the presence of rabies. When the microscopical examination proves Negri-negative or questionable, no time should be lost in taking samples of the cerebral cortex, cerebellum, and Ammon's horn on each side of the brain, plus a sample from the medulla-brain-stem. These should be pooled in the emulsifier in preparation for the mouse inoculation test described in section 4, page 56. It is quite important to achieve complete representative sampling of all those parts mentioned on each side for pooling, since there is often great variability in the virus distribution through the brain.

Antibacterial Agents for Contaminated and Decomposed Specimens

It is often difficult to obtain animal brains from the field which are bacteriologically sterile. The head may have been in transit for a long

time, or picked up long after death, or shot, or clubbed in the head. Also, the cause of death may have been a bacterial encephalitis.

Intracerebral injection of bacteria may cause the death of inoculated mice in one, two, three, or more days, before the rabies virus, which may be present in the inoculum, has had its full incubation period. On the other hand, injected mice may live long enough for rabies incubation to be complete, may pass through the typical rabies symptoms of tremors, paralysis, and prostration, followed by death, and may show typical Negri bodies and many bacteria in their brain smears.

When bacterial contamination is suspected, for example, if the animal brain is decomposed or when many bacteria are demonstrated on the original brain smears, it is best to treat the brain suspension with an antibacterial agent before inoculating it into mice. Of the following agents, penicillin and streptomycin [4] will give the best results. If these antibiotics are not available, any of the other agents may be used—e.g.:

(1) *Glycerol.* Place the brain specimen in pure glycerol for 48 hours.

(2) *0.5% phenol.* Make up 0.5% phenol in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(3) *1 : 5,000 thiomersal (merthiolate).* Make up a 1 : 5,000 solution of thiomersal in physiological salt solution. This is used as the diluent for making up the 10% tissue inoculum. Hold for 6 hours or overnight. If held for 6 hours, keep the suspension at room temperature. If held for over 6 hours, keep the suspension overnight in the refrigerator.

(4) *10% ether.* Place one part ether in ten parts tissue suspension, shake in a stoppered bottle and leave at room temperature for two hours; use a sterile cotton plug to allow for evaporation of ether.

Caution If all the ether has not evaporated, its intracerebral injection will cause the almost instant death of the mice.

(5) *Penicillin and streptomycin.* Add 500 units of soluble sodium penicillin G and 2 mg streptomycin per ml of tissue suspension. Allow to stand for 30 minutes at room temperature before injection. This amount is usually enough, but for very heavily contaminated brains or salivary glands, as many as 1,000 units of penicillin and 3 mg of streptomycin may be used.

In order to help detect the possible presence of contaminating bacteria, a portion of all tissue emulsions should be cultured in dextrose infusion

[4] Johnson, H. N. [illegible] 81, 382

broth or similar media, and streaked on a blood-agar plate. The recommended amount is about 0 1 ml of emulsion in 3 ml of broth. Incubate overnight at 37 5°C.

Early deaths (1-3 days) among the infected mice may be attributed to the presence of contaminating bacteria if the cultures show moderate to heavy growth, and if many bacteria are found in the brain smears of the dead mice.

DESCRIPTION OF PLATE

Fig. 1 is reproduced from Rivers, T. M., ed. *Viral and rickettsial infections of man*, 2nd ed., Philadelphia, London, Montreal, 1952, by courtesy of J. B. Lippincott Company; fig. 2 is reproduced by courtesy of Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., USA, fig. 3-8 were kindly supplied by Professor P. Lépine, Institut Pasteur, Paris.

Fig. 1 Sellers' stain, showing three large Negri bodies with "Innerkörperchen"; the small round cells stained red are erythrocytes (Magnification ×900)

Fig. 2. Fox: non-specific inclusion body in cytoplasm of degenerated neuron (anterior horn cell). Note homogeneity and lack of inner structure of inclusion body. (Mann stain: magnification ×1,440)

Fig. 3. Street rabies—I. The cell right of centre shows the difference between the nucleolus (stained violet-red inside the nucleus) and the Negri body (in the same cell just below the nucleus). Other Negri bodies can be seen in the cytoplasm of the cell at the 10 o'clock position. (Mann stain: magnification ×680)

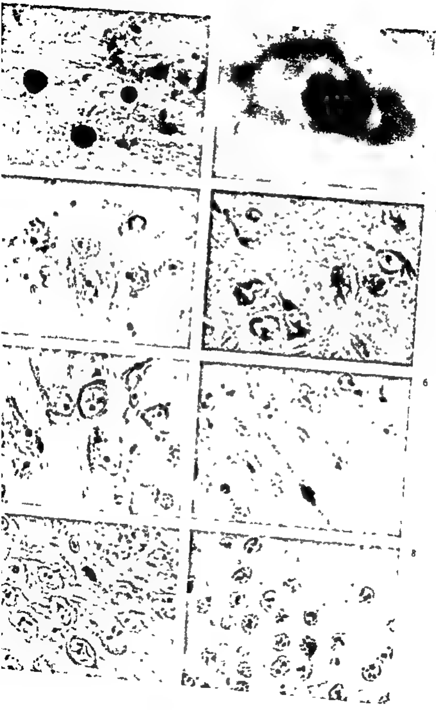
Fig. 4. Street rabies—II. Note Negri body in cytoplasm of neurons below centre of figure. (Giemsa stain: magnification ×680)

Fig. 5. Street rabies: typical Negri bodies, several showing internal structure; many micro-bodies present; several, situated at the prolongations of the neurons, appear to be extra-cellular. (Lépine fuchsin-safranine-blue stain: magnification ×680)

Fig. 6. Fixed-virus rabies—I. Typical oxyphilic nuclear degeneration characterizing fixed-virus lesions. (Mann stain: magnification ×680)

Fig. 7. Fixed-virus rabies—II. Similar type of oxyphilic nuclear lesion in a neuron in the outer layer of Ammon's horn. (Giemsa stain: magnification ×680)

Fig. 8. [illegible] of nuclear degeneration resulting in fixed-virus rabies les[illegible] polychrome bodies fill the nucleus of the affected neu[illegible] [illegible]in stain: magnification ×680)



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4

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8

HISTOPATHOLOGICAL DIAGNOSIS

nciple, the histopathological diagnosis of rabies consists in recog- the presence in the animal of acute encephalomyelitis which scribed to a specific agent, namely the rabies virus

e animal has died from rabies, it is normally easy to detect the lesions, *if the animal has been killed, death may have occurred* the appearance of specific lesions (Negri bodies). Thus, all animals .h the cerebrospinal axis (brain, medulla, ganglia) shows the slightest of lesions, particularly infiltrations, should be regarded as suspect, atter how small the lesions may be.

fter correct removal, the brain should be carefully dissected. Smears npressions should first be examined for Negri bodies (see section 2, [illegible] ibedding by a rapid method. At least six samples should be examined, rresponding respectively to the cornu ammonis (both sides), the cerebral ortex (motor area), the cerebellum, the medulla, and a ganglion (gasserian r upper cervical).

The sections should be examined for

(*a*) Signs of meningo-encephalomyelitis, i.e., meningitis, meningeal infiltration, perivascular cuffing, parenchymatous infiltration, formation of encephalitic nodules (Babès' nodules), and ganglionar infiltration with satellitosis and neuronophagy (lesions of van Gehuchten and Nélis (see fig 1 and 2)

These lesions may be detected by any staining method (haematein-eosin, polychrome blue) They show the existence of encephalomyelitis and enable a *probable* positive diagnosis to be reached

(*b*) Specific lesions : The different types of neurons should be examined for Negri bodies and lesions of fixed-virus rabies

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* Contributed by Pierre Lépine, Virus Section Institut Pasteur Paris, France

FIG. 1. NORMAL GASSERIAN GANGLION

Ganglioneurons with a single layer of satellite cells

FIG. 2. RABIES : INFILTRATED GASSERIAN GANGLION

Infiltration, satellitosis and neuronophagia (lesion of van Gehuchten & Nélis)

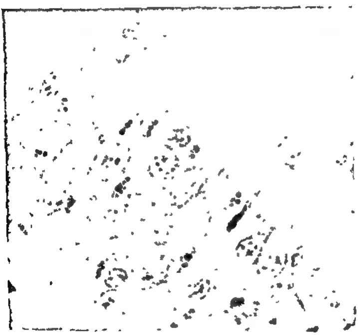
FIG. 3 RABBIT STREET RABIES

Mann stain magnification ×2,000

Negri of layer)

The Negri bodies are found especially in the central pyramidal layer of Ammon's horn (see fig. 3) and the hippocampus, in the lower loop and the middle layer of the ganglioneurons of Ammon's horn and, less frequently, in the neurons of the cerebellum (motor areas), and of the medullary nuclei. They may be present in very large numbers in the ganglia but are generally small in size.

FIG 4 RABBIT : FIXED-VIRUS RABIES



Lépine (fuchsin-safranine-blue) stain magnification x 770

Polychromatic degeneration of neurons in outer layer of Ammon's horn

The lesions of fixed-virus rabies are found exclusively in the middle zone of the external layer of the cells of Ammon's horn (see fig 4 and 5). They always co-exist in a varying proportion with the lesions of street-virus rabies.

These lesions can be detected only by special staining methods (Mann, Giemsa (see colour plate, fig 4, facing page 40), Sellers, etc). Their presence enables a *definitely* positive diagnosis to be made.

FIG. 1. NORMAL GASSERIAN GANGLION

Ganglioneurons with a single layer of satellite cells

FIG. 2. RABIES : INFILTRATED GASSERIAN GANGLION

Infiltration, satellitosis, and neuronophagia (lesion of van Gehuchten & Nélis)

FIG. 3 RABBIT : STREET RABIES

Mann stain magnification ×2 000

Negri bodies in neurons of Ammon's horn (inner layer)

occipital bone, followed by two more cuts, one on each side, along the temporal bone in prolongation of the cuts already made. Join up the saw-cuts by means of bone-cutting forceps, and lift up and push backwards the top of the skull In the case of very large animals (large dogs, cows, etc.) a different method is preferable: make a longitudinal saw-cut on each side of the midline at about 1 5 cm from it, and join up these cuts by one or two transverse saw-cuts above the orbits and at the occiput so that the calvaria can be removed in two symmetrical pieces

Once the calvaria has been removed, use fresh instruments to open the meninges, making use of serrated dissection forceps and a pair of fine, sterile scissors The operation is performed by making an incision in the meninges, starting from the median region, along and on each side of the longitudinal sinus, next, a second incision is made perpendicular to the first and the meningeal flaps pushed upward and backwards Again change instruments; cut through the medulla with a scalpel as low as possible and lift up the brain proceeding from back to front, and successively severing the pairs of cranial nerves At the end of the operation the brain is toppled over forwards into a large sterile Petri dish where it rests on its upper surface In hot weather, or if the brain is soft (cadaveric brain), it should be cooled to $+5°C$ (refrigerator) to give it a firmer consistency before dissection.

Examination of the brain

Note whether or not there is congestion of the cerebral vessels or exudate in the meninges, etc. Dissect the brain as follows:

(*a*) With a brain knife separate the two hemispheres longitudinally, after having detached the cerebellum and the medulla.

(*b*) Look for the hippocampus and the cornu ammonis. This may be done in two ways

(1) Cut across the brain transversely, starting from the base behind [illegible]

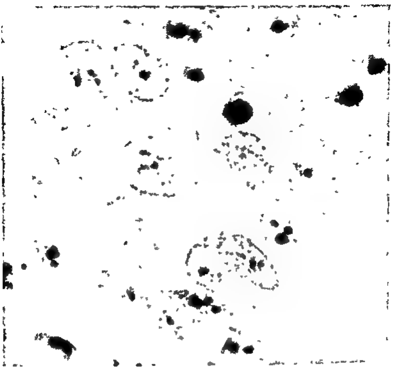
can easily be removed

(2) Alternatively, a longitudinal incision may be made externally in the posterior third of each cerebral hemisphere about 1.5 cm from the midline, the incision is continued through the grey matter and the white matter until the third ventricle is reached in the form of a narrow fissure [illegible]

tricle [illegible]

later [illegible]

FIG. 5. RABBIT : FIXED-VIRUS RABIES



Lépine (fuchsin-safranine-blue) stain magnification ×1,850

Detail of affected neurons pyknosis of cellular bodies, with multiplicity of polychromatic nuclear debris

BRAIN REMOVAL, AND PREPARATION OF MATERIAL

Large Animals (Dogs, Cattle)

The animal is firmly secured to the autopsy table or, better, the head is separated from the body and strapped to a wooden block hollowed out for the purpose The operator and his assistants should wear thick rubber gloves to protect their hands. Make an incision on the midline of the skull through the skin, push aside the flaps of skin, and reflect the muscles and fascia as far as the base of the skull, proceeding from the crown to a horizontal line passing through the eyes. Saw transversely through the skull at eye level and make a symmetrically placed saw-cut through the

of eversion which completes the clearance of the field of operation. The Farabeuf and bone forceps are then laid aside. With the fine forceps and the scissors the meninges are cleared aside and the anterior part of the brain sectioned at the olfactory lobe, the medulla is severed behind the cerebellum, and after the brain has been raised in order to cut the optic chiasm, it is placed in a Petri dish where it is dissected

With the brain lying on its dorsal surface in the Petri dish, the ventral surface facing upwards, cut the cerebral trunk at the peduncle; then cut through the encephalic mass obliquely along a transverse slanting plane, starting from the optic chiasm and going towards the convexity, parallel to the posterior surface of the hemispheres and to the cut surface of the cerebral trunk. A second cut made in the same way, parallel to the first and from 5 mm to 8 mm behind it, gives a transverse section of the brain which includes the gyrus hippocampi and the cornu ammonis as well as the basal optic ganglion—areas of choice for the detection of Negri bodies, in addition to the cortical motor area (see fig 6 and 7, and colour plate, fig 5, facing page 40) A transverse section of the cerebellum makes it possible to examine Purkinje's cells and the peduncular region. Finally, a slice cut from the end of the cerebral trunk gives a section of the medulla To reach the gasserian ganglion, cut through the petrosal bone with the bone forceps at its insertion into the sella turcica; make the cut surface gape open by everting the temporal bone downwards and outwards The gasserian ganglion is easily recognized from its whitish and nodular aspect and its almost fibrous consistency. The ganglion is carefully removed, freed from fragments of bone, placed on a piece of filter paper, and immersed in the fixing agent with the other samples

Guinea-pig, hamster

The animal is secured to the autopsy tray and the head thoroughly freed from skin The skull is rapidly flamed With the second pair of large sterile scissors, open the skull by means of four incisions encircling the skull cavity, the first one joining the two orbits and the other three made successively at the sides and the occipital bone Remove the brain with the fine forceps and scissors and dissect it as described above for the rabbit It is more difficult to locate the gasserian ganglion than with the rabbit, but, with care, this can be done successfully

Mouse

The mouse is pinned, ventral surface downwards, on a sheet of cork, the limbs being spread out and fixed First of all the four paws are fixed, the base of the tail is secured with a fifth pin, and a sixth is passed through

Cut transverse sections 1-2 mm in thickness from each hippocampus Take similar samples from the cerebral cortex (motor area), the cerebellum, and the medulla, and immediately submerge them in the fixing agent selected.

When impressions are to be examined at least six slides (two for each hippocampus, one for the cerebral cortex, and one for the cerebellum) should be carefully inspected before deciding that the results are negative. If the results are negative, however, histological examination and animal inoculations are carried out.

Preparation of the tissue samples for histological examination

If the tissue is soft and difficult to section, prepare pieces of filter paper slightly larger than the tissue sample to be collected. Apply the piece of filter paper to the cut brain surface, grasp the edge of the filter paper with fine forceps held in the left hand, and with the right make a cut with a scalpel parallel to the filter paper and 2 mm or 3 mm from it, so as to remove the piece of brain, which is immediately submerged in the fixing agent together with the fragment of filter paper to which it is adhering

Removal of material for inoculation

In operating, care should be taken to put on one side fragments aseptically removed from the same areas (cortex, hippocampus, cerebellum, medulla) for use in animal inoculation (see section 6 (A), page 77) If the brain is received in good condition and can be assumed to be sterile, the fragments are removed before any examination is made. When the brain is infected, the fragments may be removed at any time and antibiotics added, as described in section 2, page 38.

Small Animals (Rabbit, Guinea-pig, Hamster, Mouse)

Rabbit

The animal, resting on its ventral surface, is attached to the autopsy tray, with the head at the edge of the tray. Using a serrated dissection forceps, the scalpel and the scissors, completely scalp the head from the nape to the muzzle, removing the ears and the upper eyelids. Moisten the exposed surface of the head with iodized alcohol and rapidly flame it with a bunsen burner Holding the muzzle of the animal with a Farabeuf forceps in the left hand, open the brain pan with three cuts of the bone forceps Make the first two in the front part of the head, from each orbit to the midline with an upward and outward movement of eversion, opening the brain pan (parietal and temporal bones) in two flaps, to the right and to the left The third cut is made at the occiput, with a backward movement

of eversion which completes the clearance of the field of operation. The Farabeuf and bone forceps are then laid aside. With the fine forceps and the scissors the meninges are cleared aside and the anterior part of the brain sectioned at the olfactory lobe; the medulla is severed behind the cerebellum, and after the brain has been raised in order to cut the optic chiasm, it is placed in a Petri dish where it is dissected.

With the brain lying on its dorsal surface in the Petri dish, the ventral surface facing upwards, cut the cerebral trunk at the peduncle; then cut through the encephalic mass obliquely along a transverse slanting plane, starting from the optic chiasm and going towards the convexity, parallel to the posterior surface of the hemispheres and to the cut surface of the cerebral trunk. A second cut made in the same way, parallel to the first and from 5 mm to 8 mm behind it, gives a transverse section of the brain which includes the gyrus hippocampi and the cornu ammonis as well as the basal optic ganglion—areas of choice for the detection of Negri bodies, in addition to the cortical motor area (see fig. 6 and 7, and colour plate, fig. 5, facing page 40). A transverse section of the cerebellum makes it possible to examine Purkinje's cells and the peduncular region. Finally, a slice cut from the end of the cerebral trunk gives a section of the medulla. To reach the gasserian ganglion, cut through the petrosal bone with the bone forceps at its insertion into the sella turcica; make the cut surface gape open by everting the temporal bone downwards and outwards. The gasserian ganglion is easily recognized from its whitish and nodular aspect and its almost fibrous consistency. The ganglion is carefully removed, freed from fragments of bone, placed in a piece of filter paper, and immersed in the fixing agent with the other samples.

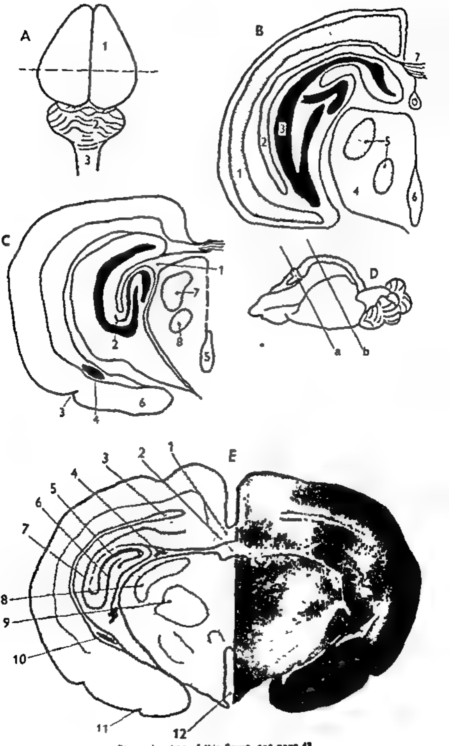
Guinea-pig, hamster

The animal is secured to the autopsy tray and the head thoroughly freed from skin. The skull is rapidly flamed. With the second pair of large sterile scissors, open the skull by means of four incisions encircling the skull cavity, the first one joining the two orbits and the other three made successively at the sides and the occipital bone. Remove the brain with the fine forceps and scissors and dissect it as described above for the rabbit. It is more difficult to locate the gasserian ganglion than with the rabbit, but, with care, this can be done successfully.

Mouse

The mouse is pinned, ventral surface downwards, on a sheet of cork, the limbs being spread out and fixed. First of all the four paws are fixed, the base of the tail is secured with a fifth pin, and a sixth is passed through

FIG. 6. SECTIONS OF RABBIT AND MOUSE BRAIN



For explanation of this figure, see page 49

the anterior extremity of the muzzle which is firmly stretched forwards. Remove the skin from the head. Treat the skull with iodized alcohol and flame it very gently with the pilot flame of the bunsen burner. Open the skull with the fine scissors, commencing by joining the orbits, then cutting laterally through the skull fairly low, and finally pushing back the flap thus obtained. Sever the medulla and the chiasm. Remove the whole of the brain mass and place it in a Petri dish. Separate the cerebellum. Cut through the brain transversely at the optic chiasm and along a plane parallel to the posterior surface of the brain, so as to obtain a cross section which is placed in the fixing agent, the anterior portion being reserved for smears and inoculations. Fix also a section of the cerebellum.

If it is wished to examine a ganglion, the upper part of the spinal cord should be detached and removed together with the upper cervical ganglion.

EMBEDDING, STAINING, AND EXAMINATION FOR NEGRI BODIES

Rapid Mercury Sublimate Fixing Agent[4]

We recommend the following mixture for rapid fixation of the nervous system, equal volumes of glacial acetic acid, acetone, and saturated solution of sublimate ($HgCl_2$) in absolute alcohol.

The mixture is made in advance and keeps perfectly well. Prepare the saturated solution of sublimate in absolute alcohol in hermetically sealed bottles, hasten saturation by keeping in the incubator at 37°C. The sections should be treated with Lugol's solution or iodized alcohol before staining, in order to remove the sublimate. Mixtures containing other components, particularly formol, give distinctly inferior results, as do mixtures into which water or hydrated alcohol has been introduced in order to slow down

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Key to fig. 6 (page 48)

A Brain (mouse, rabbit). 1 cerebrum, 2 cerebellum, 3 medulla oblongata

B Section of normal mouse brain. 1 cerebral cortex, 2 lateral ventricle (choroid plexus), 3 Ammon's horn, 4 mesencephalon, 5 inner nuclei of grey matter, 6 median ventricle, 7 corpus callosum

C Section of normal rabbit brain. 1 third ventricle, 2 Ammon's horn (middle layer), 3 rhinal fissure, 4 choroid plexus, 5 infundibulum, 6 gyrus of hippocampus, 7 lateral geniculate ganglion, 8 ventral nucleus of thalamus

D Rabbit brain after removal from cranial cavity. Inverted, lateral aspect. Left—anterior portion, centre—temporal and parietal lobes, right—cerebellum. Section is performed anterior to a (through optic chiasm) and posterior to b, the portion a-b is immersed in fixative

E Rabbit brain. transverse section a-b. 1 corpus callosum, 2 third ventricle, 3 lateral ventricle, 4 choroid plexus, 5 Ammon's horn (middle layer), 6 Ammon's horn (inner layer), 7 Ammon's horn (outer layer), 8 lateral geniculate ganglion, 9 ventral nucleus of thalamus, 10 lateral choroid plexus, 11 rhinal fissure, 12 infundibulum

fixation. Tissues other than those of the nervous system are hardered by this fixing agent.

Rapid Method for Histological Embedding

For slices of brain tissue 1 mm in thickness, fixation is complete in 15-30 minutes. The tissue is transferred directly to absolute alcohol, with which it is treated for 20-30 minutes in two baths, followed by two charges of toluene and two of paraffin, each lasting 15 minutes. Including the time necessary for cutting and staining the sections, the preparation is ready for microscopical examination from three and a half to four hours after autopsy of the animal.

Embedding Using Dioxan

It is sometimes advantageous to use a solvent for paraffin which is also miscible with water. The number of manipulations is reduced and for fairly thin tissue fragments there is a gain of time. The dioxan technique is suitable for use in this connexion.

Fix the tissue in thin slices, not exceeding 5 mm in thickness. In principle, any fixing agent can be used. It is preferable, however, to employ Bouin's dioxan mixture [8] as given by Lison:

dioxan saturated with picric acid . . .	8.5 volumes
commercial formol . . .	1 volume
glacial acetic acid . . .	0.5 volume

Next embed without washing, following the procedure given below: [1]

dioxan I . .	1 hour
dioxan II	1 hour
dioxan III	2 hours

followed by

paraffin I	15 minutes
paraffin II	*45 minutes*
paraffin III	2 hours

Use fresh paraffin for embedding.

Take care to keep the dioxan in well-stoppered bottles (risk of chronic poisoning accompanied by anaemia caused by the vapour if the dioxan is kept in open bottles), and ensure that it remains anhydrous by adding pieces of CaO.

Staining of the Negri bodies by Mann's Method

This classic method gives sections which are permanently stained, with very fine differentiation of the Negri bodies. It is an excellent demonstration method, but it requires time and a certain knack for full success.

The procedure is as follows:

Prepare the mixture at the time of use:

methyl blue (not methylene blue), 1% aqueous solution	18 ml
1% aqueous eosin solution	. . 23 ml
distilled water	. . 49 ml

Stain for 24 hours at laboratory temperature, or for 6-14 hours at 38°C (in this case first treat the section with alcohol-formol to render the gelatine insoluble since otherwise the sections come loose).

Was, first with tap-water, next with absolute alcohol (rapidly)

Differentiate in alcoholic caustic soda solution

1.5% solution of caustic soda in alcohol	1 ml
absolute alcohol	30 ml

until the section is stained pink (about 10 minutes) As soon as this stage is reached, wash the preparation well with tap-water. The section should take on a sky-blue colour, if not, treat it with water containing acetic acid (2 drops of acetic acid in 40 ml of distilled water) for 1 minute

Dehydrate rapidly (absolute alcohol); treat with xylol, mount in balsam.

Result · Negri bodies, vermilion red, nucleoli of the neurons, violet red, chromatin, blue; cells, dark blue; stroma, pale blue, erythrocytes, pink (see colour plate, fig 3, 6, and 8, facing page 40).

On substituting phloxin B for eosin in the same proportion, the preparations obtained are less attractive (purplish-blue or mauve background instead of sky-blue) but the inclusions (Negri bodies) are more numerous and more striking

Staining of the Negri Bodies by the Fuchsin-Safranine-Blue Method[3]

After being well fixed, the tissue is embedded in paraffin, cut into thin sections, and freed from the paraffin. Stain for 10 minutes with the following mixture

(1) basic fuchsin	1 g
50% alcohol . .	200 ml
(2) 0 2% aqueous solution of safranine	

Mix equal parts of (1) and (2) (drop-bottle, the mixture is fairly stable and keeps for some time).

Discard the stain, cover the section with alcohol-acetone (equal parts) to remove excess stain, wash rapidly: the section is coloured red. Stain for from 15 seconds to one minute with Unna's polychrome blue (10% dilution) or with permanganate blue prepared by Stévenel's method[11] and used undiluted. Discard the stain: the section is deep violet in colour. Differentiate in alcohol-acetone for a few seconds: the section becomes blue; immediately wash the preparation in running water (tap-water) to remove the excess of blue stain, and again treat with alcohol-acetone; next, without washing, commence dehydration by shaking in a Borrel tube filled with absolute alcohol. The remaining stain is removed from the section which becomes differentiated, taking on a pink-lilac tint varying in paleness according to the thickness of the section. Rapidly complete dehydration in absolute alcohol, carefully remove the alcohol in several changes of xylol, and mount in balsam.

Result: stroma, very pale pink with nerve fibres a deeper pink; neuroglia and leukocytes, purplish-blue; neurons, light blue; chromatin, deep purple with the nucleolus a vivid red; pathological formations particularly well shown: nuclear inclusions and oxyphilic substances, vivid pink; Negri bodies, poppy-red to mauve pink, with the internal structure lilac (see colour plate, fig. 5 and 7, facing page 40).

Detection of Negri Bodies with the Fluorescence Microscope

This method makes use of the fluorescence of the Negri bodies when they have been impregnated with a fluorochrome and subjected to light with a wavelength of 4,000 Å.[5] When equipment for fluorescence microscopy is available this is a rapid and sure method of rabies diagnosis.

(1) Fix the brain tissue and embed in paraffin as usual. Fine sections are made which are mounted on slides and treated successively with xylol, absolute alcohol, and water to remove the paraffin.

(2) Next, stain by immersion for 30 minutes in an aqueous 0.2% solution of thioflavine S. Without washing it, treat the preparation for a few seconds with absolute alcohol and then with toluene. Mount in balsam between the slide and the cover-slip, applying as thin a layer of balsam as possible. It should first be ascertained that the balsam does not contain substances which become fluorescent in ultraviolet light.

(3) Next examine with the fluorescence microscope, using a filter to block wavelengths longer than $\lambda = 5,150$ Å. Locate the cornu ammonis

under low magnification and examine with high magnification, using glycerol or liquid paraffin as immersion liquid

The Negri bodies immediately stand out because of their vivid fluorescence (see fig. 7 and 8) and brilliant azure-blue colour. The background of the preparation is pale-yellow, cellular protoplasm brilliant yellow, nucleoli and erythrocytes pale-blue.

FIG. 7 STREET RABIES : AMMON'S HORN (MIDDLE LAYER)

Mann stain magnification ×600

Appearance under white light, many Negri bodies

Method of Stovall & Black

A popular staining method used in the USA is that described by Stovall & Black [12] using acetone fixation and a sequence stain. The following description is taken from Lillie.[8] A variant of this method is also given by Lillie

"1 Stain 2 minutes in a 1% alcoholic solution of ethyl eosin (C I No 770, sodium ethyl eosinate) adjusted to pH 3 0 with N/10 hydrochloric acid

2 Rinse in water

3 Stain 30 seconds in 0 23% methylene blue in 22% alcohol buffered to pH 5 5 with the acetic acid-sodium acetate buffer

4. Then differentiate in 0.38% acetic acid in water (13 drops in 60 cc) until sections are brownish red.
5. Wash, dehydrate, and clear.
6. Mount in balsam.

Results: Negri bodies are brownish to pure red, nucleoli pale blue, other structures pink.

FIG. 8 STREET RABIES: AREA OF BRAIN, TISSUE BLOCK, AND CELLS IDENTICAL WITH THOSE OF FIG. 7

Mann stain magnification ×800

Appearance, under same magnification but with fluorescent light, of Negri bodies fixed by thioflavine and standing out clearly, because of their fluorescence, against the dark background of the preparation

* *A variant of this method* which we have used successfully.

1. In 90 cc 100% alcohol or 94 cc 95.5% alcohol, 3.25 cc 1% acetic acid, and water to make 100 cc, dissolve 950 mg ethyl eosin. Stain in this for 2 minutes.
2. Then wash in alcohol and in water.
3. Counterstain formalin material in 0.5% methylene blue in 25% alcohol, alcohol-fixed material in alum hematoxylin.
4. Differentiate in 0.25% acetic acid for 2 to 5 minutes.
5. Wash, dehydrate, and clear.
6. Mount in balsam.

Other Staining Methods

Among the innumerable methods which have been recommended for staining Negri bodies the following also give good results:

(1) Lentz's method -[2] this is a variation of Mann's method.

(2) Gallego's method,[1] using acid ferric chloride as mordant, followed by staining by Ziehl's method and with picro-carmine.

(3) Romanowsky-Giemsa method: a buffered Giemsa following the technique of Lillie & Pasternack.[7]

(4) Schleifstein's method:[10] a combination of Sellers' method with the rapid dioxan-embedding method

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Section 4 *

MOUSE INOCULATION TEST

The mouse inoculation test, in spite of its simplicity, depends greatly on the accuracy of its performance for dependable results.

Choice of Mice

Strain

Usually white mice of any breeding strain may be considered suitable. Preference should be given to the Swiss albino strain since it is very susceptible to rabies virus and it is easy to maintain the breeding stock in the laboratory. If the Swiss albino stock is not available, however, almost any breed of mice, except grey wild mice, can be used, because a genetically resistant strain has not yet been found. Grey wild mice should be excluded, not because of insusceptibility but because of the difficulty in restraining these animals in cages during the observation period

Age

Mice of all ages are susceptible to intracerebrally introduced rabies virus. However, it is easiest to inoculate, maintain, and observe mice which are 21-35 days old (8-12 g weight) at the time of inoculation.

Sex

Mice of both sexes are equally susceptible to rabies virus It is inadvisable to keep the older mice of the same sex in one cage since the older animals, particularly the males, are apt to kill each other in fights before the observation period is completed

General health

It is imperative that the animals chosen to be inoculated be in good health It is important to know the history of the breeding colony, and it is advisable to inspect the animals closely before inoculation.

* Contributed by Hilary Koprowski, Assistant Director, Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., USA

Ectoparasites on the animal, ruffled fur, and unmistakable signs of diarrhoea, should disqualify the animals immediately. If the mice are sent to the laboratory from a considerable distance, it is advisable to postpone inoculation for a minimum period of three days in order to let them rest and become adjusted to changed conditions. In such cases it may be equally important to leave a few animals uninoculated in order to observe the death-rate among "normal" mice as compared with inoculated animals.

Preparation of Supposedly Infectious Material to be Inoculated

Choice of tissue

Either the brain or the salivary-gland tissue of a suspectedly rabid animal may be used for virus isolation Detection of the virus is more frequently possible in the brain than in the salivary gland However, from the epidemiological and epizootiological points of view, it is important to examine the salivary glands for the presence of virus

Although it is relatively immaterial which part of the brain tissue is chosen for the preparation of the suspension, preference may be given to Ammon's horn, the cerebellum, and parts of the cortex. When salivary-gland tissue is chosen, the submaxillary glands should be considered as those most likely to show the presence of rabies virus Also, when salivary-gland tissue is used, it is always advisable to mince it before grinding.

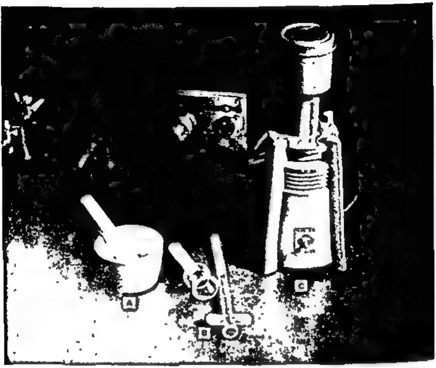
Grinder

The choice of grinder depends to a certain degree on the amount of tissue available If more than 3-4 g of material are available, a small Waring blendor should be preferred over any other type of grinder (see fig 1) However, if the amount of tissue is less than 3 g (which is more usually the case), or if a Waring blendor is not available, the following grinders or grinding devices may be suitable in the order of preference listed

(a) *TenBroeck grinder* (see fig 1) This grinder is a handy tool, easy to assemble, manipulate, clean, and sterilize, but only brain tissue may be used in it since salivary-gland tissue is too tough for it to grind properly There is a slight disadvantage with respect to its fragility If improperly used, it may break while being manipulated, and the grinder may be easily damaged during cleaning, sterilizing, etc From the point of view of personal safety, the Waring blendor and the TenBroeck grinder, if properly used, have some advantage over the other grinding devices

(b) *Mortar and pestle* (see fig 2) This is a time-honoured method of grinding which has one advantage, namely, that with the help of an abrasive (e g , sterile sand) even the toughest tissue can be properly ground up

FIG. 1. GRINDERS FOR PREPARATION OF TISSUE



A = Mortar and pes'e B = TenBroeck grinder C = Waring blendor (small size

The mortar and pestle cannot be operated under as sterile conditions as ca[n] the Waring blendor or the TenBroeck grinder, it can, however, be easil[y] cleaned and sterilized, and stands wear and tear for a long period of time

(c) *Makeshift devices* If Waring blendors, TenBroeck grinders, o[r] mortars are not available, the following makeshift tools may be considered

(i) *Concave dish and paint brush* Any concave dish and medium-size[d] paint brush which can be sterilized may be used for grinding brain tissu[e] alone The suspension can be made quite simply and is as homogeneou[s] as one prepared in a mortar and pestle, although more time is required The brush should be scrupulously cleaned and properly sterilized afte[r] use

(ii) *Mincing with scissors.* If no grinders at all are available, the possibility of mincing the tissue curved scissors may be considered. This may be atch-glass kept inside a Petri dish. It is the since some pieces of tissue will always ting, etc

FIG 2. PREPARATION OF TISSUE SUSPENSION BY EMULSIFICATION WITH MORTAR AND PESTLE

By courtesy of United States Department of Health, Education and Welfare Public Health Service Communicable Disease Center (US DHEW-PHS-CDC)

Diluent

The choice of diluent may be left to the user but it is preferable to use an isotonic salt-concentration. However, mice have been known to withstand easily an intracerebral inoculation of sterile distilled water The following diluents may be considered in order of their availability.

(*a*) *A physiological salt solution containing varying amounts of animal serum (10%-50% concentration)* This is by far the most common diluent used It should be carefully ascertained, however, that the donor animal has never been vaccinated against rabies It is therefore usually advisable to avoid the use of dog, cat, or cattle serum Normal sheep serum seems to possess some "antiviral" properties which are absent in rabbit serum. Thus, if it is available, the rabbit should be considered as the first source of blood It is preferable to inactivate the serum for 30 minutes at 56°C before using it as a component of the diluent Diluent containing serum can be sterilized only by filtration through bacteria-retaining filters

(*b*) *Other diluents*:

(i) skimmed milk

(ii) albumin solution in a balanced salt concentration

(iii) physiological salt solution with distilled water

These are not particularly recommended, although rabies virus seems to be relatively non-susceptible to the inactivating action of salt solution, in contrast to such viruses as eastern or western equine encephalomyelitis

Note: *If a tissue suspension has been frozen and stored, a 50% serum: water solution should be the preferred diluent.*

Bacterial sterility

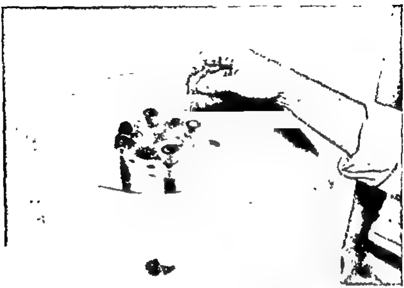
There is no need to add antibiotics to suspensions of *brain tissue* if the material has been handled with reasonable precautions at the autopsy, and dispatched in a sterile container. A 50% glycerol solution is highly recommended for preserving the material since, in addition to its preserving qualities, it exerts a strong bacteriostatic action (see section 2, page 38) If there is any doubt, however, it may be safer to add enough streptomycin and penicillin to the suspension to obtain a concentration of 2 mg of streptomycin and 50 units of penicillin per 1 ml of the final suspension. If antibiotics are added, it is best to let the suspension stand for at least 30 minutes before inoculating the animals.

It is always advisable to add antibiotics to suspensions of *salivary glands* Regardless of the addition of antibiotics, the salivary-gland tissue suspension should always be cultured for possible bacterial contamination. Beef-infusion broth, thioglycollate medium, and blood-agar are considered to be good culture media for this purpose. If bacterial growth is observed, an attempt should be made to identify the bacterial agent. If the results of the mouse inoculation test are equivocal (see page 67) it may be advisable to test the pathogenicity of the bacterial contaminant for mice injected intracerebrally

Concentration of infected tissue in the suspension

This is optional. If the suspension is for storage, it is advisable to prepare a 20% suspension by weight. The weight of the tissue in grams multiplied by 4 gives the correct amount of diluent in millilitres to be added However, if the suspension is to be used for inoculating mice intracerebrally, a 10% suspension by weight should be given preference. Either dilute the 20% suspension by adding an equal volume of diluent, or prepare the suspension by multiplying the weight of tissue in grams by 9 in order to obtain the required amount of diluent in millilitres.

FIG. 3. CENTRIFUGATION OF TISSUE SUSPENSION



1,000 r p m for 5 minutes

By courtesy of US DHEW-PHS-CDC

FIG 4 APPEARANCE OF CENTRIFUGED TISSUE SUSPENSION

By courtesy of US DHEW-PHS-CDC

In attempting to isolate certain strains of rabies street virus, the "self-sterilizing neuro-infection" phenomenon may be very occasionally suspected, and in such rare cases it is advisable to dilute the tissue suspension more than 10% (see page 67-68).

Centrifugation and filtration

If the equipment is available, it is advisable to centrifuge the tissue suspension for 5 minutes at 1,000 revolutions per minute (r.p m.) in order to remove the gross particles (see fig. 3 and 4). However, if no centrifuge is available, it is perfectly feasible to inject mice intracerebrally with a 10% uncentrifuged brain suspension. Salivary-gland suspensions, if uncentrifuged, must be filtered through one or two layers of sterile gauze in order to prevent the death of the animals from trauma.

Inoculation of Mice

Choice of syringe

Syringes should be chosen which can measure accurately 0 03 ml (single mouse dose). Thus $^{1}/_{4}$-ml tuberculin syringes should be considered first, followed by ½-ml or 1-ml tuberculin syringes For intracerebral inoculation, 27- or 26-gauge (0.40-0.45 mm) needles 1-1.5 cm long should be selected. Larger-gauge needles cause trauma to the brain substance.

Anaesthesia

It is strongly recommended that mice be anaesthetized before they are injected. Inhalation of ether is the best form. A battery jar with a specially fitted wire bottom may be used for the purpose (fig. 5). If no such device is available, pentobarbital sodium injection should be considered. It is a good procedure to have the work-table used for mouse inoculation drawn well away from the wall, so that an assistant can etherize the mice from a position opposite the operator.

Inoculation technique (see fig. 5)

There are as many methods of inoculation as there are investigators. The present author is right-handed and prefers the following technique.

Anaesthetized mice are laid on the left side, the legs pointing towards the inoculator With the thumb of his left hand, the inoculator supports the lower jaw of the animal, with his index finger behind the skull of the animal Pressure should be very slight in order to avoid the asphyxiation and death of the animal In his right hand, the inoculator holds the syringe

FIG 5. INTRACEREBRAL INOCULATION OF MICE

in a horizontal position parallel to the table surface and perpendicular to the head of the mouse, with the needle pointing towards the inoculator. With a quick thrust, the needle is pushed through the skull of the animal at the place which can best be described as the apex of an imaginary angle of which the arms point to the animal's right eye and right ear. The needle easily penetrates through the bone and should then be further inserted for about 0 1-0 2 cm into the brain tissue When the inoculator uses a 1 5-cm needle, he should be careful not to penetrate too far since he may then end by giving the injection into the base of the skull The plunger is pushed to the next 0 03-ml mark and the needle is then gently withdrawn. For intracerebral inoculation, move the inoculated mice away from the syringe hand ... the inoculated mice to the ... h may result in catching a

The inoculated mice are immediately placed in a can or box previously prepared and identified by a tag bearing the mouse-group number or any other particular identification mark. If a large series of inoculations is made, it is worthwhile checking the number of living mice in each series

after the entire experiment is finished. If any animals are found dead, an equal number of living animals should be inoculated and added.

Under no conditions should the same syringe be used for inoculation of two different suspensions. If an adequate number of sterile syringes is not available, each syringe should be boiled between inoculations, and care should be taken to let it cool before filling it with inoculum.

It is well always to use the same rigid precautions to establish good habits in virus work. For example, the rapid emptying of a syringe into a pan of water will produce an aerosol which can cause inhalation infection in the operator, or the animals with which he is working, if the disease can be transmitted by such exposure. Virus may be spread from table to hands, and if these are not washed properly it is possible to contaminate subsequent specimens during grinding of tissue in a mortar—for example, virus may fall from the hands or sleeves into the mortar. Mice laid on the table after etherization may awake and have to be put back in the ether jar, thus contaminating it. Should other studies be carried out using the same ether jar, virus may be deposited on the heads of normal mice and be carried into the brain by the inoculation procedure. The work-table must be considered contaminated until washed with soap and water. Dilute bichloride of mercury is a good disinfectant for table tops. Cresol and phenol solutions are no better than water for the most common virus contaminant of mouse tissue, namely, the mouse encephalomyelitis virus, and several other viruses; phenol has no appreciable viricidal action on rabies virus

When inoculation of all the mice is finished, the syringe and needle may be rinsed in water, provided this is done gently with the point of the needle well below the surface of the water in the sterilization tray. Sterilize all syringes and needles by boiling for five minutes.

Observation of Inoculated Mice

Although rabies virus will only rarely cause signs of illness in mice before the fifth day after intracerebral inoculation, it is advisable to check mice daily, beginning with the first post-inoculation day. The number of mice found normal, sick, or dead is recorded on a mouse-history card (see fig 6) which remains on file as a permanent record of the experiment. The observation period should extend for a minimum of 21 days after inoculation. Only rarely will rabies virus be detected in the inoculum later than 21 days after the inoculation.

The following signs may be noted

(*a*) Roughing fur. (*b*) tremors when held in the air by the tail with a pair of forceps. (*c*) lack of co-ordination of hind legs—note gait when

placed on table and made to move; (*d*) paralysis, (*e*) prostration (near death). Letter symbols denoting the different signs are recorded each day on the mouse-history card.

FIG. 6. MOUSE-HISTORY CARD

No Date Virus

Strain Passage Preparation

Volume Dilution

DATE																						
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1																						
2																						
3																						
4																						
5																						
6																						
Checked by																						

Age of Mice Route

LFE 542

Deaths in mice occurring 24-48 hours after intracerebral inoculation are attributable to causes other than rabies virus (trauma, bacterial contamination, other viruses). For diagnostic purposes, one or two mice may be sacrificed each day, beginning on the fifth day, and a search made for Negri bodies. Frequently, an early diagnosis is thus obtained, particularly in instances where certain strains of street virus might take between 1 and 3 weeks to kill the mice

Note Clinical signs of illness in inoculated mice cannot be considered characteristic of rabies Although signs of paralysis 5 days or more after inoculation may give grounds for suspecting the presence of rabies virus, the same signs may be observed in numerous other viral, bacterial, and protozoal infections which involve the central nervous system of the mouse. Definite evidence of the identity of the virus is obtained with the serum-virus neutralization test (see section 5, page 69)

Further Passages of Infected Material

If it is desired, brain tissue from mice which have succumbed to infection after inoculation with the original virus may be made into a suspension as described above and stored, or used in neutralization tests, or inoculated into another group of mice

thereby exposing the brain. The brain is removed with curved scissors (see fig. 7) and is transferred to a sterile Petri dish. A thin section of the brain is cut out, just anterior to the cerebellum, and is transferred to a wooden tongue-depressor or a paper towel. A clean microslide is then pressed lightly against the cut surface of the section; the pressure should be sufficient to create a slight spread of the exposed surface against the glass slide. Negri-body stain should then be applied to the slide.

Complications of the Mouse Inoculation Test

Bacterial contamination of inoculum

If bacterial contaminants caused the death of the mice in spite of the addition of antibiotics, and if the original suspension was preserved, the following methods may be tried in an effort to overcome the interfering action of the bacteria.

(a) *Filtration through bacteria-retaining filters*: The supernatant liquid of a suspension centrifuged at 1,500 r.p.m. for 15 minutes should be used for this purpose. Since rabies virus is a fairly large particle and since, in general, the concentration of virus in specimens submitted from the field is not very great, the virus may be lost in the process of filtration.

(b) *Dilution method*: Sometimes the suspension can be diluted beyond the end-point of bacterial contamination with retention of viral activity, but this happens very rarely.

(c) *Prolonged storage*. In some instances, it is easier to combat bacterial contamination after the tissue suspension has been stored for a period of time either at freezing temperatures or in glycerol (see pages 38 and 60).

(d) *Parenteral inoculation*. The Syrian hamster, which is the animal most susceptible to parenteral infection, may be chosen for this purpose. Mice are relatively insusceptible to parenteral infection with rabies.

Presence of two viruses

This is particularly confusing if the second virus has pathogenic properties similar to those of rabies. Again, intracerebral or parenteral inoculation of animal species other than the mouse may be attempted, particularly in view of the extremely wide host-range of rabies virus.

Self-sterilizing neuro-infections" or interference phenomenon

In certain instances, either because of the properties of a particular strain of rabies virus or because large amounts of inactive virus particles

may interfere with the living virus, it may be necessary to dilute the inoculum ten- or a hundredfold, or even more. There is no rule to determine when this should be done. However, if failure to isolate rabies virus is consistently encountered in the same species of animal in a particular geographical area, the possibility of an interference phenomenon should be seriously considered, and tissue suspensions should be diluted beyond 10% concentrations.

SERUM-VIRUS NEUTRALIZATION TEST

If properly executed, the serum-virus neutralization test constitutes one of the best proofs of identity of rabies virus. Although the intracerebral type of neutralization test is less sensitive than a test in which injection is carried out in mice parenterally, it is impossible to apply the latter test in identifying rabies virus, since the virus has very low invasive power when injected parenterally in mice. This test will be described as though attempting to identify a virus recently isolated from the field. However, the technique may be applied to any neutralization test routinely performed in the laboratory.

Choice of Mice

The same criteria should be used as those described for the mouse inoculation test (see section 4, page 56)

Preparation of Virus Suspension

Usually the first mouse passage of a virus in the laboratory can be used as the source of infected material. However, in some instances the concentration of virus is too low and the test will fail, because too small a number of mice injected with a mixture of virus and *normal* serum will die of the infection. In such cases, the second or third laboratory passage in mouse brain should be used. The preparation of the mouse-brain suspension should follow the technique described in section 4, page 57, except that it may be more convenient to prepare a 20% suspension by weight of infected tissue. Also, the use of 10% normal-serum : physiological-salt-solution or 10% normal-serum : distilled-water-solution as diluent may give more uniform results than other diluents.

Storage of Virus

For certain technical reasons it may be advisable to store the virus before its use in the neutralization test. Several methods may be recommended for the preservation of the rabies virus. The best is to keep the

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morning and have food removed from the cages the night before so that clear serum will be obtained. Immunization of guinea-pigs should be carried out with guinea-pig brain virus The use of mouse-brain virus may result in the production of antibodies to mouse encephalomyelitis virus if this is present as a contaminant in the mouse-passage strain. This may lead to a false interpretation of the results of the specificity test. Immune serum from guinea-pigs, prepared as described above, may also be used for the complement fixation test. Rabbits are also suitable animals for preparing immune serum (see section 6 (B), page 85).

Performance of the Test

Serum

Prepare two rows of sterile 12 × 75-mm tubes, each row containing five tubes. With a wax pencil mark each set of tubes 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Mark one set with letter N (normal serum) and the other with letter I (immune serum) In each tube of the set marked N, place 0 2 ml of normal serum, measured from a 1-ml pipette calibrated in 0 01 ml. Do the same with the set marked I, using immune serum. (Use separate pipettes for each serum)

Virus

Prepare a set of 12 × 110-mm tubes, also marked 10^{-1} to 10^{-5}. Leave the first tube empty, into each of the remaining tubes pipette 2.7 ml of diluent (preferably 10% normal serum physiological-salt-solution. Into the first, empty, tube marked 10^{-1}, pipette the previously prepared 20% suspension of infected mouse brain Transfer 0 3 ml of this suspension into the tube marked 10^{-2} and mix it well with the diluent, using another pipette (1-ml), by blowing the fluid back and forth about 12 times. Following that, transfer 0 3 ml into the tube marked 10^{-3}, mix well, etc , as before.

Measure 0.2 ml of the contents of the tube marked 10^{-1} into the tube containing normal serum (N-10^{-1}) and then 0 2 ml into the tube containing immune serum (I-10^{-1}). Follow the same procedure with the remaining dilutions of virus, using a fresh pipette for each dilution

Shake the rack well and place it in either a water-bath or an incubator set at 37°C for one and a half hours.

Note In preparing serial dilutions it is essential to use a fresh clean pipette for each transfer After transferring the desired quantity to a subsequent tube, the fresh pipette should be used for mixing, and then for transfer to the next tube A routine should be established whereby the tube to which the virus has been added is set apart from the other

tubes in the rack before mixing. Mixing is accomplished by filling and emptying a 1-ml pipette about 10 to 12 times; avoid frothing. A not uncommon error with inexperienced technicians is to transfer without mixing, or to skip a tube and so fail to carry on any virus.

Under no circumstances should titration be attempted if sufficient pipettes are not available for changing with each dilution, because the titration will be meaningless. By titration with a single pipette it is possible to obtain infection in mice with 10^{-17} dilutions, etc., because a little tissue virus keeps coming off the inside of the pipette. This may be illustrated by diluting methylene blue in tenfold steps using a single pipette.

Inoculation of Mice

Remove the tube-rack from the incubator or water-bath and place it in a tray containing water and ice-cubes. Inject the contents of each tube intracerebrally into six mice (0.03 ml per mouse) following the technique described in section 4, page 62. The series of mice receiving the immune-serum : virus mixtures should be inoculated first. One tuberculin syringe may be used for the immune-serum series and one for the normal serum. However, in that case start the inoculations with 10^{-5} dilution and proceed backwards to 10^{-1} dilution. While changing dilutions, always rinse the syringe with the "new" dilution before filling it up. Be certain to have six living mice injected with each dilution at the end of the test.

Observation of Inoculated Mice

Follow the procedure described in section 4, page 64. Terminate the test on the 21st day after inoculation unless the presence of contaminating mouse encephalomyelitis virus is suspected (see page 73). If uniform mortality ratios are observed, the test may be terminated on the 14th day.

Calculation of Results

Hypothetical results of a typical test are summarized below:

Serum	*Mortality ratio of mice inoculated with serum and dilutions of virus* 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	*LD_{50} titre of virus*	*Mouse LD_{50} of virus neutralized*
Normal	6 6	6 6	6 6	4 6	0 [illegible]	$10^{-4.25}$ *	—
Immune	6 6	2 6	0 6	0 6	0 6	$10^{-1.75}$	316 †

* See section 11, page 111 for logarithmic calculations.

† Antilogarithm of 2.5, the difference between 4.25 and 1.75.

Interpretation of Results

If 100 LD_{50} or more of virus is neutralized by the serum, the identity of the virus is established. In case of equivocal results it is desirable to repeat the test. Sometimes neutralization of 75 LD_{50} of virus may be considered to be a sufficient criterion of identification.

If the rabies virus has been lost and replaced by another virus the titre will be the same in the control and rabies-immune serum groups. Viruses isolated or maintained in mice are liable to be contaminated by mouse encephalomyelitis virus, and this virus may be passed in series with rabies virus. Most strains of mouse encephalomyelitis virus are apt to produce symptoms somewhat earlier than the rabies fixed virus, that is, on the second or third day. Mice showing symptoms at this time should be suspected of harbouring mouse encephalomyelitis virus, and brain tissue should be taken for microscopical study and for subpassage. So-called "highly invasive" strains of mouse-passage rabies virus which overwhelm immunity produced by vaccination with rabies vaccine must be checked for the presence of mouse encephalomyelitis virus. A single subpassage through rabbits will remove the mouse disease.

The inoculated mice should be held for at least 30 days, as mouse encephalomyelitis virus, if present, may produce paralysis three to four weeks after inoculation. It is difficult to isolate virus from mice that develop paralysis at this time if they are infected with the mouse encephalomyelitis virus. The mouse encephalomyelitis virus is maintained best by subpassage at three-day intervals. The mouse disease virus is not apt to be present in high titre.

Testing of Half-Log Dilutions of Virus

For certain studies it may be advisable to include half-log dilutions of virus. When preparing the usual tenfold dilutions of virus (1 ml plus 9 ml diluent) set up alternate [illegible]

1 part in 3.16 or half-log). Do not mix the half-log dilutions until the tenfold dilutions have been prepared. Then take a clean pipette and, beginning with the highest dilution of half logs, transfer and mix successively throughout the series to the most concentrated tube, using the same pipette. It is also possible to mix the half-log dilutions in a flask with rotation agitation without using a pipette.

Modification of the Test: Dilutions of Serum with Same Virus Concentration

If the titre of the virus is known beforehand, the test may be modified by the use of a constant dose of virus representing approximately 100 LD_{50} per mouse dose when mixed with an equal amount of serum. The procedure is similar to that described above, except that the serum is to be diluted instead of the virus. This test saves mice, since it is not necessary to use more than one dilution of normal serum. The immune serum may be diluted tenfold or fivefold, depending on its previously determined neutralizing potency. This is a more sensitive test than the virus dilution test but it has more application as a standard laboratory procedure than as a method of identification of an agent of unknown titre. It is particularly useful in determining relative titres or potencies of serum samples.

Part II

METHODS OF VACCINE PRODUCTION

Section 6 *

PHENOLIZED VACCINE: SEMPLE TYPE

A. SHEEP-BRAIN VACCINE: METHOD OF INSTITUT PASTEUR, PARIS

General formula for vaccine

[illegible]

Rabies strain employed

The strain employed is the Institut Pasteur fixed virus (Louis Pasteur strain) isolated on 19 November 1882, which has been maintained solely in the rabbit since that date. On its 90th passage this strain was used for the first human immunization (6 July 1885) and since then it has been constantly employed at the Institut Pasteur in Paris for immunization purposes. The behaviour and characteristics of the strain are periodically examined and have formed the subject of much research. At the present time, titration of the strain in the rabbit shows that a 1 : 15,000 dilution of the fixed virus kills rabbits six times out of seven, whereas a 1 : 16,000 dilution kills them five times out of ten, a 1 : 17,000 dilution rarely kills them, and a 1 : 18,000 dilution, never.

Since 1 April 1952 the Pasteur strain, which formerly was continually passed in the rabbit, has been regularly passed once a month. For passages of the strain, at least two rabbits with a minimum weight of 2 kg are inoculated intracerebrally with 0.25 ml of a 1 [illegible] suspension of brain tissue prepared from the preceding passage (the brain is kept in glycerol at +5°C). The rabbits, which are paralysed on the sixth day, are sacrificed by bleeding on the seventh day. The brains, which are extracted under sterile conditions, are subjected to bacteriological sterility tests, histological examination for the detection of typical fixed virus lesions (see colour plate, fig. 6, 7,

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* Contributed by Pierre Lépine (Chief) and R. Bequignon, Virus Section, Institut Pasteur, Paris, France (A) and Martin M. Kaplan, Chief Veterinary Public-Health Officer, Division of Communicable Diseases Services, World Health Organization, Geneva, Switzerland (B)

and 8, facing page 40) and titration of the virus by inoculation of a dilute suspension of fresh virus into rabbits.

Immediately after removal, the brain is placed in pure, sterile glycerol and dispensed into wide-necked bottles. Labels indicating the number of the rabbit and of the passage are placed on all bottles, which are kept in the refrigerator at between +2°C and +5°C.

By 1 June 1953, the Pasteur strain had undergone 1,908 passages since its isolation.

Preparation of inoculation virus

For inoculating sheep the last passage of the strain is used, i.e., a brain from the regular series of passages, kept in glycerol in the refrigerator for more than a week but less than a month. A fragment of brain is removed, rinsed under sterile conditions with sterile distilled water, and weighed. It is then ground up in a chilled mortar so as to obtain finally a 1 : 10 emulsion of brain (by weight) in physiological salt solution. This constitutes the inoculum. Each sheep is injected intracerebrally with 0.5 ml of the inoculum at one point of the brain, using the technique described below.

Preparation of sheep

For vaccine purposes only young sheep in good health are used. The average weight of the animals is 42 kg, and the brain of such animals weighs about 80 g. The animals are shorn and kept under observation for

FIG. 1. TABLE FOR RESTRAINING SHEEP FOR INTRACEREBRAL INOCULATION

FIG. 2 SITE AND TECHNIQUE FOR TREPHINING OF SHEEP

three weeks and all animals which do not appear to be completely normal are eliminated

On the day before inoculation the head of the sheep is shaved and the skin then disinfected by vigorous brushing with a brush steeped in cresyl solution The skin of the skull is then painted with tincture of iodine This last procedure, which is carried out in the morning and evening, is repeated on the morning of the next day At the time of inoculation the shaven skull of the animal has thus been disinfected with cresyl and with three applications of tincture of iodine.

The animal is taken to the inoculation laboratory and immobilized on a special table (see fig 1) The skin is held taut by an assistant, and trephining performed, without a previous incision, using a trephining drill with a point 13 mm long and 2 mm in diameter (see fig 2) The point of the instrument is directed towards the lateral-external region of the left hemisphere of the brain (lateral face of the frontal lobe) As mentioned above, each animal receives 0 5 ml of inoculum in a single injection

Several sheep are always inoculated during the same session, the usual number being eight sheep per session The sheep show the first symptoms of rabies seven to eight days later, and all are sacrificed on the ninth day when they are already lying on their side but still alive The animals are

FIG. 5 EXTRACTION OF SHEEP BRAIN

Left—sterile Pyrex-glass jar in which brain is kept at low temperature

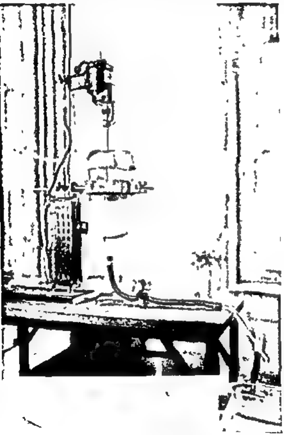
cooled by a jacket filled with crushed ice. Two brains at a time (about 160 g of brain) are placed in the apparatus. Grinding is performed without diluent, with the machine running at full speed for a period of four minutes. A small amount of sterile physiological salt solution is then added. After having allowed the blendor to run for three minutes to ensure that the mixture is homogeneous, samples are taken for the control culture and the final dilution is made in large Pyrex containers holding five litres.

The diluent consists of sterile physiological salt solution which is phenolized at the time of use. To do this, a 5% solution of pure phenol is made with physiological salt solution and is diluted immediately before use in the proportions necessary to obtain the final concentration of vaccine. The proportions are calculated according to the weight of the brains as follows

Sheep brain	1 g
Sterile physiological salt solution	16 ml
5% phenol in physiological salt solution	4 ml

The vaccine mixture is agitated and then left for 24 hours in an oven at 22°C. On removal from the oven, the vaccine is immediately dispensed into 5-ml ampoules. It is essential for the mixture to be absolutely homogeneous so as to ensure that the quantity dispensed into each ampoule is

FIG. 8. DISTRIBUTION INTO AMPOULES OF ANTIRABIES VACCINE



Note turbo-stirrer immersed in vaccine suspension which ensures dispersion of virus during distribution

exactly the same For this purpose it is necessary to keep the vaccine stirred during the process of filling into ampoules In order to do this, special adapters and shafts of an electric turbo-stirrer are used. The stainless-steel shaft (about 35 cm long), turbine, and adapter can be detached

from the driving motor and sterilized in advance. The turbo-stirrer has the advantage over ordinary stirrers in that it ensures a much more homogeneous mixture without frothing. In this way unlimited quantities of vaccine can be filled into ampoules with the certainty of rigorous homogeneity and, consequently, of correct dosage (see fig. 6).

The vaccine ampoules are kept for at least a week at between +2°C and +5°C before being sent on to the packing service. During this minimum period of storage, which completes the inactivation of the virus, the final control of the vaccine is carried out.

Final control of vaccine

Before being distributed for use, the vaccine undergoes the following tests:

(1) Sterility test, as described above.

(2) Inactivation test, by intracerebral inoculation of the rabbit.

(3) Potency test. For this test either Habel's method (see sections 11 and 12, pages 112 and 116) is used or the rabbit control test described by Béquignon & Vialat (see section 14, page 125). A rabbit weighing 2 kg receives the vaccine subcutaneously for ten consecutive days at the rate of 2 ml per day. On the 30th day it should be protected against the intracerebral inoculation of a fixed-virus dilution of 1 : 10,000.

Furthermore, from time to time vaccine samples are subjected to an additional control consisting of the assay of the dried extract and of the phenol, as well as determination of the pH.

Period of use

The vaccine can be used during a period of five months dating from its distribution, provided that it is kept at a temperature of between 2°C and 5°C throughout this time. If low-temperature storage cannot be guaranteed, the period of use of the vaccine is decreased to two or three months, according to the climate of the region where it is employed.

Phenolized vaccine should not be frozen at any time during its preparation or storage.

Method of use

The vaccine is supplied in boxes each containing 20 5-ml ampoules, representing a complete treatment. In the case of severe bites and bites on the face, 30 injections are recommended instead of the 20 which constitute the standard treatment. The volumes injected are reduced by half in the case of children aged less than 10 years.

B. RABBIT-BRAIN VACCINE: METHOD RECOMMENDED TO MEET REQUIREMENTS OF UNITED STATES NATIONAL INSTITUTES OF HEALTH *

The following procedure is recommended to meet the stringent requirements of the National Institutes of Health (NIH) in the United States of America [1] Horses, sheep, and goats can be and are used instead of rabbits for the preparation of vaccines, particularly for veterinary purposes (see section II (A), page 77).

Formula of vaccine

The vaccine comprises 20% inactivated rabies fixed virus in phosphate-buffered saline (pH 7), 0 25% phenol, 1:10,000 thiomersal (merthiolate). The NIH requirement for vaccine to be used in human beings stipulates that each dose of the final vaccine should contain not less than 2 0 ml of a 5% brain-tissue suspension, or its equivalent in another concentration. The dose of a 20% suspension would therefore be 0 5 ml. With respect to preservative, in addition to the final concentration of 0.25% phenol, phenylmercuric borate 1 : 12,500 or thiomersal 1 : 10,000 may be used. Phenol concentration in the finished vaccine should not exceed 0 25% for vaccines containing 10% or less of brain tissue, and should not exceed 0 4% for vaccines containing more than 10% brain tissue.

Virus strain

The Pitman-Moore strain,[2] or other suitable fixed virus strain which through experience has given satisfactory results, should be used

Maintenance of virus strain

Virus passage for vaccine production is always through rabbit brain. A seed virus is used for any convenient period (usually not more than three months) as long as full potency is maintained as shown by mouse titration. A 25% virus suspension in buffered distilled water (pH 7) is quick-frozen and stored, preferably in a CO_2 chest (−70°C) but at least at below −15°C.

* The procedure described is based on successful [illegible]

Seed virus must be of sufficient strength to kill all mice injected intracerebrally with 0.03 ml of the rabbit-brain suspension in a dilution of not less than 10^{-5}.

The purity of the virus strain is tested periodically by titration in mice against specific antirabies hyperimmune serum which has proved protective against street virus (see section 18, page 139).

Production of seed-virus 25% suspension

Rabies-infected rabbit brains are weighed and then emulsified in 3 parts of buffered distilled water (pH 7). Emulsification is effected in a small (200-ml capacity) Waring blendor for 3 minutes; 1-ml amounts are placed in 5-ml ampoules, sealed, quick-frozen in dry-ice and alcohol, and stored, preferably at dry-ice temperature (–70°C), but at least at below –15°C.

Sterility

0.5 ml of 25% virus suspension is inoculated into each of two tubes containing 30 ml of sodium thioglycollate medium or other suitable media for a 14-day sterility test, including a subculture incubated for seven days (see page 90).

Virus titration

At least two intracerebral mouse titrations of 10^{-5}, 10^{-6}, and 10^{-7} dilutions are performed. The test period is 14 days, and all mice receiving the 10^{-5} dilution should die.

Inoculation technique

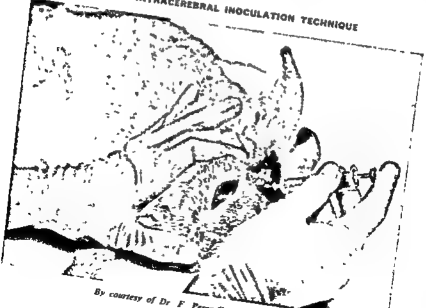
From the 25% suspension of seed virus a final 1 : 1,000 dilution of virus is made in 2% normal serum in distilled water. The diluted virus suspension is centrifuged in an angle centrifuge (approximately 1,000 g) for 15 minutes, and the supernatant fluid is used as the inoculum. A fresh dilution is made for each day's inoculation, and is tested for sterility in thioglycollate medium.

Healthy rabbits weighing 3-4 kg are firmly secured and 0.2 ml of the inoculum is introduced by a sterile 2-ml syringe and 23-gauge 3/8-inch (0.65 mm × 1 cm) needle through a puncture made in the skull 1.5 cm from the outer canthus of the eye. This is usually into the right hemisphere only, although two inocula of 0.05 ml each can be used for the two hemispheres. A stainless-steel awl or a punch can be used to make the puncture (see fig. 1 and 2).

FIG. 1. USE OF PUNCH FOR INTRACEREBRAL INOCULATION

By courtesy of Dr F Perez-Gallardo Madrid

FIG 2 INTRACEREBRAL INOCULATION TECHNIQUE



By courtesy of Dr F Perez-Gallardo, Madrid

Progress of disease in rabbits

On the 4th day rabbits show marked signs of paralysis, which increase rapidly to complete paralysis on the 5th (or occasionally as late as the 6th) day.

Killing of rabbits

Moribund rabbits which have shown rabies symptoms for at least 24 hours, and are completely paralysed except for the chest muscles, are killed by intravenous injection of 10 ml of air into the marginal vein of the ear. Death occurs in from one to two minutes. The brains from freshly killed rabbits only are used for vaccine production; animals already dead from rabies or other causes are not used.

Autopsy

To guard against the use of unsuitable rabbits, autopsies are performed after brain removal. The brains of rabbits showing the presence of coccidiosis elsewhere in the body are not used because of the chance that coccidiosis rather than rabies may have caused paralysis. A record is made of all autopsy findings.

Brain harvest

The rabbit is washed with creolin or another suitable antiseptic solution. Decapitation is performed as close to the shoulders as possible; the severed head is fastened securely into a special holder, swabbed with tincture of iodine, and taken into a "sterile room" where actual brain removal is performed.

The skin is slit down the middle of the head and folded back and the exposed top of the skull is washed with iodine from a small dropper bottle. The top of the skull is then removed with sterile bone-shears, and the brain is transferred with sterile scissors and forceps into a suitable sterile container. A small piece of the brain tissue is removed with a sterile swab and inoculated in a tube of 15 ml of thioglycollate medium for a sterility test. Brains are stored at 15°C or lower, preferably much lower, until needed for vaccine. Any brain showing bacterial contamination is discarded.

Manufacture of Vaccine: 40% Suspension

Emulsification

Frozen brains are weighed and placed in a 2-litre Erlenmeyer flask as a 40% suspension in sterile phosphate-buffered sodium-chloride solution

containing sufficient phenol to give a final concentration of 0.5% (see under *Diluent* below). After the brains are thawed, the flask is shaken by hand until they are well broken up. This partial emulsion is then trans- [illegible] ication [illegible] other [illegible] inning the emulsifier long enough to generate appreciable heat. From two to three minutes are usually sufficient for a Waring blendor to emulsify the brains adequately. The container can be surrounded with a jacket of chipped ice and salt to prevent overheating of contents

Diluent

(*a*) Phosphate-buffered sodium-chloride solution: prepare a solution containing 2 parts M/10 Na_2HPO_4 and 1 part M/10 KH_2PO_4.

To one part of this solution add four parts 0 85% NaCl of pH 7 0.

(*b*) To make a 40% tissue-concentration containing 0.5% phenol, add to phosphate-buffered saline solution 0 68% of 90% fine reagent phenol (this allows for volume of emulsified brain tissue), make a 40% weight/volume with the diluent according to weight of the brains.

Note· After phenol has been added, great care should be taken not to freeze brain-tissue suspensions, either in the concentrated 40% form or in the final vaccine dilution. *Freezing of phenolized suspensions destroys antigenicity*

Virus titre of brain-tissue pool before incubation

A sample of 40% tissue suspension is weighed (because of air content from action of the mill), diluted immediately, and titrated in mice weighing 16-20 g Dilutions of 10^{-4}, 10^{-5}, and 10^{-6}, representing dilutions of brain-tissue content, are injected intracerebrally and the titre (LD_{50}) should be at least 10^{-6} (see section 11, page 113 for calculation)

Incubation

The emulsified vaccine containing 0 5% phenol is brought to 37°C in a water-bath for 1 hour and then placed in an incubator at 37°C for about 72 to 76 hours (the longer period is used if the preliminary safety test fails) The vaccine is agitated mechanically during the entire period in the incubator

Note The period in the incubator required for inactivation will vary with different virus strains and methods of emulsification of brain tissue (fineness of particles) The time required should be established in each

laboratory through experimental trials, e.g., intracerebral inoculation of mice with suspension at 6-hour, or less, incubation intervals.

Dilution to 20% vaccine

The 40% vaccine is diluted with an equal volume of the sterile phosphate-buffered salt-solution containing 1 : 5,000 thiomersal, to make the finished product 20% tissue, 0.25% phenol, and 1 : 10,000 thiomersal

Diluted vaccine is passed through several layers of gauze or silk filter for the purpose of removing any unground particles of brain tissue. A convenient batch of vaccine is about 2 litres in volume. It is subjected to the safety and sterility tests described below, and is stored at 4°-6°C.

Sterilization

A sterility test of the finished vaccine is made in sodium thioglycollate medium or other standard media. Ten ml from each bulk container of vaccine is divided into 3 portions and inoculated into 3 bottles each containing 225 ml of medium. The test is mixed and placed in an incubator at 31°-32°C. The medium is mixed carefully after a 48-hour incubation period. Tests are observed each day for 7 days for bacterial growth. At the end of 7 days the bottles are mixed carefully, and 1 ml from each bottle is inoculated into tubes containing 40 ml of fresh sodium thioglycollate medium. Original bottles and subculture tubes are incubated for another 7 days Any vaccine showing contamination is retested, using a double volume for the test.

Safety test

Immediately after dilution and at the time of sterility testing, a 2-ml sample is taken for the intracerebral inoculation of 5 mice with a 0 03-ml dose At least 3 of the 5 mice must survive without symptoms of rabies

Final batching

The final mixture is made after the end of the preliminary safety test, or after re-incubation of the vaccine at 26°C if at least 3 of the mice tested have not survived the preliminary safety test Batches are combined in a bottle of adequate size for filling ampoules.

Samples are taken for the following tests·

Analytical assays

A sample of finished vaccine is re-incubated for 24 hours at 37°C to ensure the killing of the virus, and the following determinations are made: total solids, phenol, pH, and thiomersal.

Sterility

Test performed as described under *Sterilization*, page 90.

Innocuity

Not less than 2 rabbits and 2 mice are used Each rabbit is injected intracerebrally with not less than 0 25 ml and each mouse (weighing 18-20 g) with 0 03 ml of the vaccine containing not less than 5% of brain tissue. All test animals are observed for not less than 14 days, during which time each animal must remain free from symptoms of fixed rabies or of other central-nervous-system disease.

Final standardization to meet NIH Requirements

Potency: (see section 13, page 117).

Safety: A safety test is made on the contents of a final container selected at random from each filling of each lot, or portion of a lot The [illegible]

[illegible] death At least 2 animals of each species are used, and the observation period is not less than 7 days.

Dating

The expiration date is not more than six months after the date of manufacture or date of issue. For dating purposes, the date of manufacture may be considered as the last date of passing a satisfactory potency test The date of issue may be three months after the date of manufacture. The product is stored at 2°-5°C at all times. Freezing of phenolized suspensions should be carefully avoided

Section 7 *

ULTRAVIOLET-LIGHT-IRRADIATED VACCINE

Principles and Methods

The ability of irradiation by ultraviolet (UV) energy to cause bacterial sterilization has been known for several years, but the effectiveness of UV-inactivated microbiological antigens as vaccines is a more recent application of that knowledge.

UV energy has little ability to penetrate biological substances since it is absorbed rapidly. Therefore, for effective exposure, the material being irradiated must be presented to the incident UV rays in a very thin film. The present practical use of UV-irradiated rabies vaccine became possible only after the development of apparatus which would accomplish this thin-film type of exposure. It is also well known that as the amount of UV energy absorbed by biological material increases, the degree of chemical change in that material increases, so the length of exposure must be as short as possible while accomplishing the purpose of inactivation, otherwise breakdown of the antigen will occur. The biological effect of UV irradiation on viruses is instantaneous, for all practical purposes, and once this immediate effect has taken place, no further action ensues. There is no evidence that any secondary by-products are formed in the irradiation process which would subsequently be deleterious to the antigen.

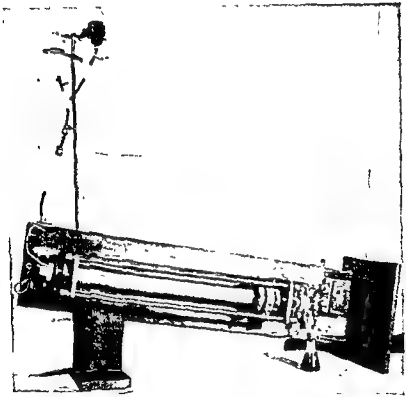
Besides the necessity for a controlled length of exposure and for the material to be irradiated in a thin film, the practical production of large volumes of vaccine requires that the apparatus used should allow for a continuous flow of the antigen suspension through the equipment. Although quartz is a good conductor of UV energy and has been used extensively for making various types of container in which an antigen could be exposed, in general an apparatus designed to eliminate the passage of the UV rays through quartz gives more efficient radiation. Ease of operation and the use of an easily available inexpensive source of UV are, of course, other desirable features of any practical irradiation apparatus.

* Contributed by Karl Habel, Chief, Laboratory of Infectious Diseases, National Microbiological Institute, Public Health Service, Bethesda, Md., USA

Apparatus

Several types of apparatus have been designed which in some way incorporate the principles listed above. Two types are available commercially in the United States of America and both have been proved to be

FIG. 1. CONTINUOUS-FLOW UV-EXPOSURE APPARATUS



A dye solution is being used for illustrative purposes

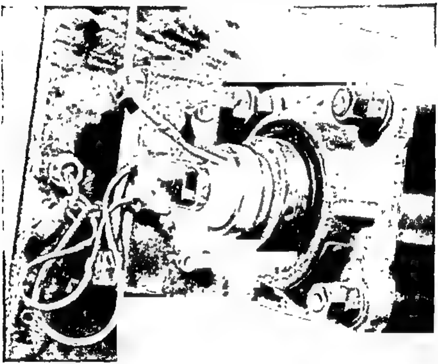
satisfactory in the production of potent rabies vaccines. The "Dill" apparatus[1] employs a standard high-energy germicidal lamp that has a long effective life, and is easily available and relatively inexpensive. The "Oppenheimer-Kettering" apparatus[2] uses a special highly efficient lamp which is more expensive.

[1] Dill Irradiator, J. J. Dill Co., 1302 Bixby Road, Kalamazoo, Mich., USA

[2] Oppenheimer Kettering UV Irradiator, Michael Reese Research Foundation, 29th Street and Ellis Ave., Chicago, Ill., USA

However, the construction of the type of apparatus of which the Dill machine is a modification can be accomplished by any modern machine-shop from basic materials that are readily available and not excessively expensive. The shop-made model used in our laboratory consists of a long hollow metal or glass tube, preferably of stainless-steel, so that the inner surface over which the virus suspension flows is very smooth and easily cleaned

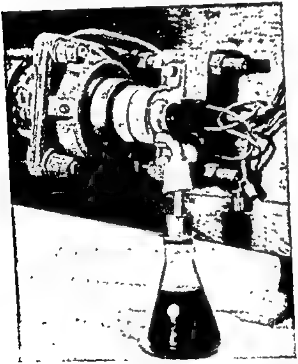
FIG. 2. INTAKE END OF UV-EXPOSURE APPARATUS



This tube is of such dimensions that a germicidal lamp suspended through the centre of it will have one centimetre of clearance from the inner wall. This tube is fitted to slide smoothly into an outer, more sturdy, tube made of brass, which is mounted in bearings at each end. These tubes are to be rotated at a speed between 500 and 1,000 revolutions per minute (r.p.m.), so a driving force must be applied. This may be accomplished by a gear- or belt-drive, but in our own apparatus it is achieved rather simply by making the outer brass tube the armature of a small AC motor of the type used in electric fans. For long runs in large-scale production this may result in greater heating of the tube

A suitably designed collecting-cup fits tightly to the discharge end of

FIG. 3 COLLECTING END OF UV-EXPOSURE APPARATUS



inner side of the rotating inside tube, where it is immediately spread in a film approximately 1 mm in thickness which progresses the length of the tube The film is thus continuously exposed to UV light and is collected through a closed connexion into a flask at the opposite end. A burette or inverted flask may be used as the original reservoir of virus suspension, and is connected to the intake-needle by rubber tubing with a valve for adjusting rate of flow inserted in the line[3] (see fig 1-3)

[3] Blueprints of this apparatus are available from the author at National Institutes of Health, Bethesda, 14 Md, USA

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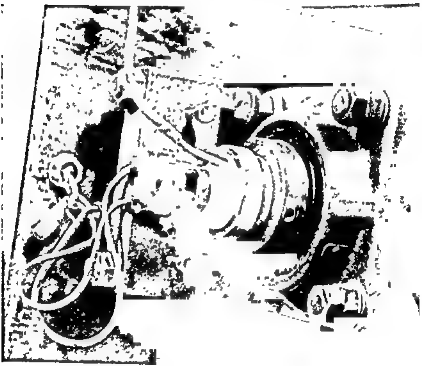
FIG. 2. INTAKE END OF UV-EXPOSURE APPARATUS



This tube is of such dimensions that a germicidal lamp suspe[illegible] the centre of it will have one centimetre of clearance from th[illegible] This tube is fitted to slide smoothly into an outer, more sturd[illegible] of brass, which is mounted in bearings at each end. These [illegible] be rotated at a sp[illegible] between 500 and 1,000 revolutions per min[illegible] so a driving forc[illegible] must be applied. This may be accomplishe[illegible] or belt-dr[illegible] but in our own apparatus it is achieved rathe[illegible] making the [illegible] brass tube [illegible] armature of a small AC moto[illegible] used in electr[illegible] fans. For [illegible] runs in large-scale productio[illegible] result in [illegible] tube

times the minimum inactivating exposure is necessary before any marked drop in antigenic potency occurs.

Virus Emulsion

All the factors of importance in rabies-vaccine production by any other method of inactivation are of equal importance for material to be irradiated. The virus titre of the original emulsion should be as high as possible, but variations of as much as one log in the titre will not noticeably change the necessary exposure-time. Since UV light will not penetrate biological materials to any appreciable depth, it is quite important that the emulsion be a uniformly fine one. To ensure this it is desirable to screen the emulsion through several layers of sterile surgical gauze or a wire screen.

In general, a 5% whole emulsion of infected nervous tissue is used with the irradiation equipment, but no difficulty is encountered in inactivating suspensions up to 10% in concentration.

Preservation and storage of irradiated vaccine

If irradiated vaccine is to be kept in the liquid state before use, a chemical preservative should be added Thiomersal (merthiolate) at a 1 : 8,000 dilution, or 0.25% phenol, appears to be superior to formalin. Irradiated vaccine, because it contains no substance in itself deleterious to viral antigen, may be kept frozen or may be dried from the frozen state. The latter process of freeze-drying will cause an immediate drop in immunizing potency, but the vaccine will then hold that potency indefinitely if sufficiently dry.

Testing of irradiated vaccine

Sterility, safety [illegible]

more consistently by the irradiation method than by any other method of inactivation There is no evidence to date that the incidence of post-vaccinal complications is any greater after the use of irradiated vaccine than with other types

Steps in an irradiation run

(1) The inner tube is cleaned with soap or detergent solution, thoroughly rinsed, and then cleaned with ether.

(2) The apparatus is assembled, and the UV lamp inserted through the tube and clamped into its sockets.

(3) The collecting end-piece, which has been autoclaved, is connected to the apparatus and, through sterilized rubber tubing, to the collecting flask.

(4) Two reservoirs, one with sterile distilled water and one with virus emulsion, are connected by a Y-tube to the rubber tubing going to the intake-needle through a flow-valve.

(5) The intake-needle is adjusted to direct flow against the inner side of the rotating tube, and is fixed in that position.

(6) The UV lamp is turned on for 5 minutes to sterilize the inside of the rotating tube, the motor of which has been started.

(7) Distilled water is run through the apparatus to wet-down the tube, and the flow is adjusted to the desired rate.

(8) The flow of the distilled water is stopped, and that of the virus emulsion started. The rate of flow is rechecked. As soon as the emulsion appears at the collecting end, a fresh collecting flask is attached, and timing of the run started. The time is again taken at the end of the run, and the volume of collected emulsion is measured.

Section 8 *

CHICKEN-EMBRYO VACCINE

At present the Flury[a] and the Kelev[a] strains of rabies virus adapted to and modified in the developing chicken embryo are used for the production of chicken-embryo rabies vaccines employed in the prophylactic immunization of dogs.[4] Vaccine made with the Flury strain has been used on a worldwide basis and therefore the description of the production procedure will be mainly confined to the Flury-strain vaccine. The same principles, however, are followed in the preparation of chicken-embryo rabies vaccine with the Kelev strain

The Flury-strain vaccine for dogs represents the 40th-50th egg-passage level of the virus. At this egg-passage level the Flury virus is fully virulent by the intracerebral route for mice, cotton-rats, hamsters, and guinea-pigs, while rabbits are more resistant. Hamsters, mice, and cotton-rats are susceptible to extraneural injection, but guinea-pigs are more resistant On the other hand, rabbits and dogs fail to show signs of infection when inoculated intramuscularly with concentrated suspensions of the virus.

Further modification of the Flury strain has been achieved by continued egg passage (180th passage), and this has resulted in a lessened pathogenicity for experimental animals while its antigenicity has apparently been retained. For the present the high egg-passage level vaccine has been recommended for use only in cattle and the low egg-passage level vaccine, described here, for use only in dogs and cats[3]

The Kelev chicken-embryo vaccine represents the 60th-70th egg-passage level of the virus At this egg-passage level the virus does not produce any signs of infection in hamsters, guinea-pigs, or rabbits inoculated either extraneurally or intracerebrally Suckling mice are, however, susceptible when inoculated intracerebrally Dogs inoculated intramuscularly with concentrated suspensions of virus fail to show signs of infection.

Preparation of seed material

At the proper egg-passage level of the virus (Flury, 40th-50th, and Kelev, 60th-70th egg-passage level), a sufficient quantity of 60% embryo

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* Contributed by A Komarov, Director, Government Virus Diseases Laboratory, Haifa, Israel

[a] These strains are available to national laboratories on request to the World Health Organization, Palais des Nations, Geneva, Switzerland

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suspension in distilled water is prepared, tested for potency (see section 15, page 128) and preserved either frozen or in its dehydrated form. This constitutes the seed material. Just before use a 20% suspension of seed material in distilled water is prepared and tested for viability as follows.

Inoculate a group of six mice intracerebrally with 0.03 ml of a 10^{-1} dilution of the seed material (use a 0.25-ml tuberculin syringe with a $^1/_4$-inch 27-gauge (0.40 × 6 mm) needle). *If the seed material contains live virus* the mice will sicken on the 6th-8th day. Should inoculated mice remain unaffected by the 9th day, discard the inoculated eggs.

In the case of the Kelev strain the viability test must be carried out in suckling mice.

Inoculation of eggs

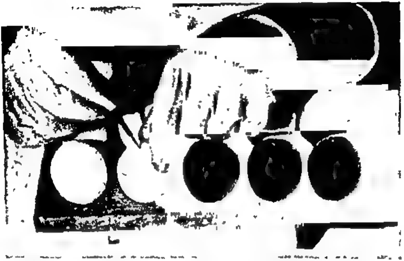
The number of eggs to be inoculated will depend on the amount of vaccine required and storage facilities. On a large production scale the average yield is 4-6 dog-doses per harvested embryo. Embryo mortality due to non-specific causes does not usually exceed 15%.

Fertile hen eggs incubated for 7 days are used. It is best to obtain fertile *incubated eggs for vaccine production from a commercial hatchery* and transfer the eggs to the laboratory one day before inoculation. On the 7th day of incubation the eggs are candled; unfertilized eggs and those with dead or weak embryos (poor vascularization, sluggishness), and eggs showing displaced air sacs are discarded. While candling, the boundaries of the air sacs are marked on the shell with a pencil and the eggs are arranged air sac uppermost in a tray (a commercial cardboard egg-tray serves the purpose well). The shell is painted at the top of the air sac with 70% alcohol, and flamed briefly.

By means of a carborundum disc attached to a rotary motor tool, or a dental drill, make two crosswise cuts, or penetrate the shell directly at the top and centre of the air sac. If this type of cutting machine is not available, pierce the shell at the indicated point by means of any sharp *instrument (see fig. 1)*.

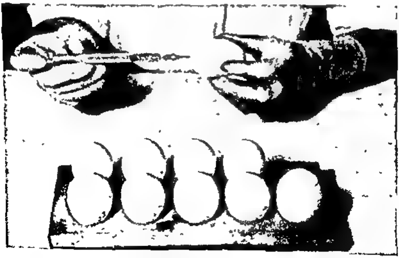
The eggs are inoculated in the yolk sac with 0.25 ml of the 20% suspension of seed material, using a syringe with a $^3/_4$-inch to 1-inch 19- to 20-gauge (6-25 mm × 0.90-1.10 mm) needle. Fill the syringe with the inoculum [illegible] the right hand parallel with the table; with the left hand take [illegible] the egg and deposit 0.25 ml of the inoculum (see fig. 2). The inoculated eggs are replaced in the tray. On completion of the inoculation the seed material

FIG. 1. PIERCING AIR SAC



By courtesy of Dr F Perez-Gallardo Madrid

FIG 2. YOLK-SAC INOCULATION OF EGG IN HORIZONTAL POSITION



By courtesy of Dr F Perez-Gallardo, Madrid

is tested for bacterial sterility, and the cut portion of the shell is sealed by means of a melted mixture of paraffin-vaseline (two-thirds paraffin, one-third vaseline), or collodion.

After inoculation the eggs are incubated at 36 5°C for a period of 9-10 days

Harvest

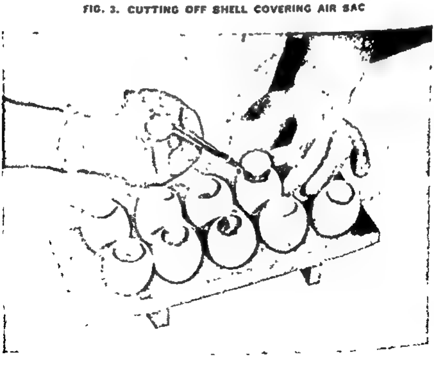
At the end of the incubation period, only living embryos are harvested *Yolk*, yolk-sac, extra-embryonic tissue, and *fluid* are *discarded*.

The eggs to be harvested are arranged in a tray, and the shells are sprayed or painted with 70% alcohol and flamed.

The live embryos are harvested by either of the following methods.

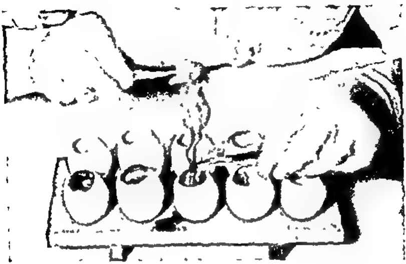
(*a*) Cut off shell over the air sac by means of sterile sharp pointed scissors (see fig. 3) Tear away the membrane, thus exposing the embryo Pass a wire hook attached to a handle under the neck of the embryo, and pull the embryo slowly upward. The embryo will be pulled out of the egg, free from the yolk and extra-embryonic material.

FIG. 3. CUTTING OFF SHELL COVERING AIR SAC



. of Dr. F Perez Gallardo, Madrid

FIG. 4. SEPARATING EMBRYO FROM MEMBRANES



By courtesy of Dr F. Perez-Gallardo, Madrid

Alternatively, lift the embryo out with one pair of forceps while separating the membranes with another (see fig. 4)

(*b*) Put on sterile rubber gloves. Crack the shell of the eggs at the centre by means of a sharp sterile instrument. Open the shell with gloved hands and pour the contents over a sterile wire gauze. The fluid and yolk should drop through the meshes into a sterile container placed under the wire gauze layer. Collect the embryos from the layer of gauze into a proper container.

Preparation of embryonic suspension

The harvested embryos are placed in a chilled weighed container. The total weight is noted and then the embryos are transferred into a chilled Waring blendor. Sufficient chilled sterile distilled water (containing 500 units of penicillin and 1 mg of streptomycin per millilitre where laboratories find that contamination occurs) is added to the blendor to prepare a 33% embryo suspension. The embryos are ground three or four times, each time for about 3 minutes, chilling the blendor between each grinding.

The resulting suspension is filtered by squeezing it through a layer of gauze into a chilled container The filtered embryo suspension is placed in 5-ml ampoules so that each ampoule contains 3 ml of 33% embryo suspen-

sion—this equals one dog-dose. Multiple doses can be used without increasing the volume of liquid, by preparing a 66% embryo suspension.

Desiccated vaccine

The embryo emulsion is desiccated from the frozen state. After desiccation, the ampoules are sealed either in vacuum or in an atmosphere of nitrogen, and stored at 4°C. In our hands, after desiccation with an Edwards apparatus, the virus titre is approximately $10^{-3.5}$ to 10^{-4}.

Bacterial safety test

Desiccated material is rehydrated and 1 ml is injected subcutaneously into 8 mice. At least 7 out of the 8 mice must survive an observation period of 14 days.

Potency test

Virus titre is distinct from the potency of the vaccine. Titres may be as low as $10^{-1.8}$ and yet the vaccine can be potent. Vaccine should not be released for use unless tested for potency.

A potency test for chick-embryo rabies vaccine is described by Koprowski (see section 15, page 128). The test consists of the intramuscular inoculation of guinea-pigs with vaccine, followed 21 days later by intramuscular challenge of the vaccinated animals with street virus. The challenge virus consists of an emulsion of rabies-infected dog salivary glands. Preliminary intramuscular titration of the virus in guinea-pigs is made and the highest dilution of the virus which kills 70%-100% of the control animals is used for challenge. In the actual test, 10 guinea-pigs each receive 0.25 ml of 5%-suspension chick-embryo rabies vaccine, and 10 animals are left as controls. Twenty-one days after the vaccination, the animals are inoculated intramuscularly with 0.1 ml of dog salivary-gland challenge virus. A vaccine of good potency will protect 70% of the vaccinated guinea-pigs.

REFERENCES

1. Komarov, A. & Horenstein, K. (1953) *Cornell Vet.* 43, 344
2. Koprowski, H. & Cox, H. R. (1948) *J. Immunol.* 60, 533
3. World Health Organization, Expert Committee on Rabies (1954) *Wld Hlth Org. techn. Rep. Ser.* 82

Part III

VACCINE POTENCY TESTS

Section 9 *

GENERAL CONSIDERATIONS

Although the need for evaluating the immunizing potencies of rabies vaccines has been recognized since the early Pasteur days, and a large volume of experimental work aimed at this goal has been carried out over the years, most laboratories producing rabies vaccine have not made a routine practice of testing their products until recently. Thus, when practical potency tests became available many laboratories were surprised to find that they had been labouring under a sense of false security since the vaccines they had been issuing for years, prepared according to well-established production procedures, were actually of very low or negligible immunizing ability. The now well-known characteristic property of all viruses, including rabies fixed strains, to mutate and change in their properties with animal passage, makes it obvious that the known requirements for the use of any one virus strain in preparing a potent vaccine 10 years ago may not be valid today.

It has always been difficult to evaluate vaccines on the basis of results obtained in human beings because of the lack of controls, the relatively small number of human cases of rabies, and the impossibility of taking into consideration all the many factors which tend to make human exposures uncomparable. Furthermore, the need is for an evaluation *before* a vaccine is released for human or veterinary use.

There would appear to be three important considerations, among others, in assessing any potency test of rabies vaccines. First, the test procedure should actually evaluate the property of the vaccine which determines its effectiveness in the prophylaxis of rabies in man or animals. Using a naturally susceptible host, the ideal test would simulate conditions of natural exposure and usual prophylactic treatment. This would mean the use of street virus introduced thro[illegible]
doses of vacci[illegible]
has, of cours[illegible]
where admini[illegible] of vaccine is started after experimental exposure of the test animal. Most tests, therefore, involve multiple doses of vaccine (as administered in man) followed by subsequent challenge with fixed virus

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* Contributed by Karl Habel Chief Laboratory of Infectious Diseases, National Microbiological Institute, Bethesda, Md USA

given intracerebrally, as being a more easily standardized type of challenge. While far from being a duplication of the situation with natural exposure and the usual use of vaccine, this type of test has been shown to reflect the relative ability of a vaccine to protect under more natural conditions.

The second important factor in a potency test is its practicability—the ease with which it is carried out, the availability of the material used, and the cost and time involved. Not all laboratories can obtain large numbers of experimental animals easily, nor can they, perhaps, support the expense of repeated tests when animal costs are high. The time factor is important, since newly prepared vaccine must be held until potency tests are complete, and this is time deducted from its period of effectiveness.

The third important factor is the standardization of the test procedure so that there may be comparability of results from one laboratory to another.

In the following sections of Part III five types of rabies potency test are described in detail. All these may be used for evaluating vaccines for both human and veterinary use, although the street virus guinea-pig test (see section 15, page 128) is specifically designed for testing the live virus chicken-embryo type of vaccine for use in animals.

The decision as to which test to use in any particular laboratory will depend on the type of information required and the facilities available. If a laboratory has limited facilities for animal procurement, and wishes merely to screen its vaccines for potency or lack of potency, then the more simple test, such as the modified Habel test in mice, should be used (see section 12, page 116). If facilities are available, and a quantitative evaluation of potency is desired as a continuing check on efficiency of production, then one of the more standardized tests is indicated. The original Habel test in mice (section 11, page 112) and the newly adapted US National Institutes of Health test (section 13, page 117) are both quantitative tests, the second being a more standardized procedure. The disadvantage of this test lies in the necessity for using a standardized vaccine and a large number of animals. The Pasteur Institute test in rabbits is also a quantitative test similar to the Habel test in mice.

Section 10 *

SOME FACTORS INFLUENCING THE STANDARDIZATION OF RABIES-VACCINE POTENCY TESTS IN MICE

For approximately the past decade the potency of rabies vaccines distributed under licence in the United States of America has been controlled by the use of an immunity-breakdown type of test in which all mice are vaccinated with the same dose of vaccine given in six injections. At the time of challenge the mice are divided into five groups, each group receiving a different tenfold dilution of virus. The potency of the vaccine is determined by calculating the number of LD_{50} of virus which will overwhelm the immune mechanism in half the vaccinated mice. An acceptable vaccine must protect mice against at least 1,000 LD_{50} of virus.

When the potency test described above is used for evaluating the same vaccines in more than one laboratory, certain variables have been recognized which could lead to vastly different potency-test results, both in the different laboratories, and in the same laboratory from one test to another. Some of these variables are: the strain of challenge virus, the technique of handling the challenge virus, and the strain of mice.

The first variable, that of the strain of challenge virus, has been studied carefully by Habel and Wright and has been eliminated by the establishment of the standard challenge virus (CVS) strain of rabies virus which is supplied to all laboratories interested in the potency evaluation of rabies vaccine.

The technique for handling the challenge virus was also standardized by Habel & Wright and a complete description has been published.[1] It has since been found that less variation is encountered in the titre of the challenge virus from test to test if, in preparing the challenge virus for each test, the virus suspension is diluted to 10% brain tissue with 2% serum in distilled water, and clarified by centrifuging for 15 minutes at a relative centrifugal force of approximately 1,000 times g. In this way all larger particles of brain tissue are removed, and a slightly opalescent supernatant fluid is obtained which contains the virus in undiminished titre.

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* Contributed by George A. Hottle, Chief, Biologics Control Section, Laboratory of Biologics Control, National Microbiological Institute, Public Health Service, *Bethesda, Md., USA*

[1] Habel, K. & Wright, J. T. (1948) *Publ. Hlth Rep. (Wash.)* [illegible], 44

The problem introduced by the use of different strains of mice is a serious one. Instances have been reported in which potency values for the same lot of vaccine have varied from 100 LD_{50} to 100,000 LD_{50} protection when different strains of mice were used. The first step which might be taken to control this variation would be the use of a reference vaccine which is put on test each time a lot of vaccine is to be evaluated. While it is desirable that the reference vaccine show the minimum acceptable potency, i.e., 1,000 LD_{50} protection, this is not essential. The potency of the reference vaccine must be determined in mice of average immunizing capacity, and the potency value obtained must be accepted by all participating laboratories. After the potency value of the reference vaccine has been established, the results of each potency test can be evaluated on the basis of the accepted potency value of the reference vaccine. Although such an evaluation will not reveal a quantitative relationship between an unknown vaccine and the reference vaccine, it is possible to get a qualitative comparison between two vaccines by means of the immunity-breakdown type of potency test.

Because of the instability of rabies vaccines, it is suggested that a dried reference vaccine be made available. This can be accomplished by suspending the infected brain tissue in distilled water, inactivating the virus with ultraviolet irradiation, and drying the vaccine from the frozen state. A dried reference vaccine prepared in this way will have retained a large part of its original antigenicity and will remain fully active for at least one year.

Suggestions for a more quantitative evaluation of two vaccines have been made on the basis of an antigen-extinction test in which groups of mice receive graded doses of vaccine. All mice are then challenged with the same dose of virus. A 50% effective dose (ED_{50}) for each vaccine is calculated as the amount of vaccine which will protect half the mice against the challenge dose of virus. Because of the variation in challenge dose of virus from one test to another, a reference vaccine is essential in this test, and potency results are always given in terms of the reference vaccine. With a test of this type the number of injections of vaccine given to the mice may be six, four, three, or even two doses. When fewer doses of vaccine are injected into the mice, the number of LD_{50} of virus in the challenge dose must likewise be reduced. With three or four doses of vaccine, the challenge dose of virus is usually 50-500 LD_{50}. With two doses of vaccine the challenge dose of virus is 5-50 LD_{50}.

The third revision of the US National Institutes of Health *Minimum requirements: rabies vaccine*, dated 3 February 1953, gives the details of the antigen-extinction type of test which will be used for evaluating all lots of rabies vaccine distributed under US licence (see section 13, page 117). In this test many of the details with regard to the challenge virus,

used with the immunity-breakdown type of test, have been left unchanged However, in the vaccination of the mice, 0 5-ml amounts of fivefold dilutions of vaccine are injected intraperitoneally, 16 mice being used for each dilution The mice receive two injections of vaccine one week apart. One week after the second injection all vaccinated mice are challenged by the intracerebral injection of 5-50 LD_{50} of the CVS virus. The mice are observed for 14 days All mice dying after the fifth day following challenge, and all mice showing paralysis, are regarded as deaths An ED_{50} for each lot of vaccine is calculated as the amount of brain tissue in milligrammes needed to protect half the mice against the challenge virus The potency of the vaccine is expressed as a factor relating to the potency of the reference vaccine, which is arbitrarily assigned unit potency. With the present reference vaccine, lot 150 C, a factor of 0 67 has been set as the minimum acceptable potency for any lot of rabies vaccine

Section 11 *

HABEL TEST FOR POTENCY

Mice

White Swiss mice, 4-6 weeks of age, and uniform in weight, are used; their sex may be selected at random, or mice of only one sex may be used if preferred.

Immunization of mice

Sixty mice receive 0.25 ml of a vaccine diluted to give a 0.5% emulsion of original wet-weight of brain. Inoculations are given intraperitoneally on Monday, Wednesday, and Friday during two successive weeks (a total of six doses). Thirty mice should be kept apart from the rest at the beginning of the immunization for use as controls at the time of challenge.

Challenge

A challenge test is performed on the 14th day from the first dose of vaccine. At this time two ampoules of the standard challenge fixed virus (see section 10, page 109, for its preparation) should be thawed out and diluted to a 10^{-1} suspension. Serial tenfold dilutions from 10^{-1} to 10^{-7} are then made, using 2% horse or rabbit serum in distilled water as diluent. It is recommended that the dilutions of challenge virus be held in an ice-water bath during the performance of the test to prevent loss of virus titre.

Groups of 10 vaccinated mice are then challenged intracerebrally with 0.03 ml of the 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} dilutions of virus in that order. A single syringe and needle may be used for all inoculations, provided the empty syringe is rinsed several times in the next dilution before being refilled.

With a new syringe the control mice are then inoculated with the 10^{-7}, 10^{-6}, and 10^{-5} dilutions of virus in order to determine what dilution represents one LD_{50}. If the challenge virus is fully active, these three dilutions will usually give a range of from 100% to less than 50% survivors. Scattered deaths at all three dilutions should be viewed with suspicion

* Contributed by Karl Habel, Chief, Laboratory of Infectious Diseases, National Microbiological Institute, Bethesda, Md., USA

For the potency test to be valid, the 50% end-point of rabies deaths should be beyond the 10^{-3} dilution in the control mice.

All mice are observed for [illegible] after the fifth day should be considered [illegible] daily and symptoms recorded. Mice surviving [illegible] showing definite symptoms of rabies should be considered as rabies deaths in potency calculations

Determination of degree of protection

50% end-points of rabies mortality are determined by the method of Reed & Muench, as illustrated in Annex 1. This gives the dilution in [illegible] where theoretically, 50% of the mice [illegible] employed [illegible] the vaccinated, the log of the number of LD_{50} protected [illegible]. To meet minimum standards, this should be log 3 or 1,000 LD_{50}.

Modification of technique for live virus or attenuated vaccines

The 6 intraperitoneal doses of 0 25 ml of equivalent 0.5% emulsion should be made starting with the least virulent of the vaccine doses and grading to the most virulent, if human doses vary as in the original Pasteur type of vaccine If all human doses are the same, then no change from the original procedure is necessary

Annex 1

PROCEDURE FOR DETERMINING 50% END-POINTS OF HABEL-TYPE POTENCY TEST IN MICE

Results of typical protocol

Dilution	*Vaccinated mice* *survived*	*rabies*	*Accumulated totals* *survived*	*rabies*	*Percentage mortality*
10^{-1}	4	6	↓ 4	↑ 20	[illegible]
10^{-2}	5	5	9	14	61
10^{-3}	3	7	12	9	43
10^{-4}	8	2	20	2	9
10^{-5}	10	0	30	[illegible]	0

Actual figures are accumulated from 10^{-1} to 10^{-5} for survivors and from 10^{-5} to 10^{-1} for rabies.

Now make a caculation thus:

$$\frac{(50\% \text{ minus the } \% \text{ next under } 50\%)}{(\% \text{ next above } 50\% \text{ minus } \% \text{ next under } 50\%)} \text{ or } \frac{50-43}{61-43} = \frac{7}{18} = 0.39$$

Now subtract this figure (0.39) from the log of the dilution which had a mortality next below 50%:

$$\begin{array}{r} 3.000 \\ 0.390 \\ \hline 2.610 \end{array}$$

This is the log of the 50% end-point for this protocol, namely, $10^{-2.6}$

Next calculate the control 50% end-point in the same way.

Dilution	*Vaccinated mice* survived	rabies	*Accumulated totals* survived	rabies	*Percentage mortality*
10^{-5}	0	10	↓ 0	↑ 17	100
10^{-6}	4	6	4	7	64
10^{-7}	9	1	13	1	7

$$\frac{50-7}{64-7} = \frac{43}{57} = 0.75$$

$$\begin{array}{r} 7.000 \\ 0.750 \\ \hline 6.250 \end{array}$$

The 50% end-point of controls is $10^{-6.2}$.

To find the LD_{50} of protection afforded by the vaccine, subtract the log of 50% end-point in the vaccinated mice from the log of 50% end-point in the controls

$$\begin{array}{r} 6.25 \\ 2.61 \\ \hline 3.64 \end{array}$$

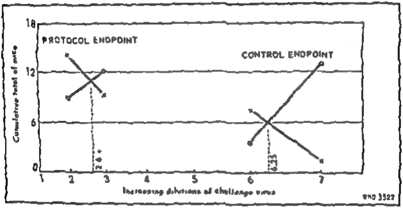
Thus this vaccine protected against $10^{3.6}$ LD_{50} or 4,366 LD_{50}. The figure should be rounded out to the nearest hundred, i.e., 4,400 LD_{50}

Graphical method for calculating LD_{50}

Approximately the same result, or one of sufficient accuracy, can be derived without fractional calculations by making use of a sheet of semi-logarithmic paper with decimal divisions. It is sufficient to know the

cumulative totals of the mice which have survived and those which have died at the doses on either side of the 50% end-point, as calculated above. The number of mice is indicated on the arithmetic scale of the sheet as an ordinate, the virus dilution being entered on the logarithmic scale as an abscissa The four points corresponding to the mice surviving and mice dying above and below the 50% end-point are plotted in this way

FIG 1 GRAPHICAL METHOD FOR CALCULATING LD_{50}



∘ = Survivals (cumulative) × = Deaths (cumulative)

against the corresponding concentrations. The concentrations of challenge virus are entered on the logarithmic scale (abscissa), the left-hand extremity of the sheet corresponding to the higher concentration, and the right-hand extremity to the higher dilution, of virus. The figures corresponding respectively to the number of mice which have died and the number which have survived are joined by straight lines The point of intersection of the two lines gives the 50% end-point, the value of which is read off on the logarithmic scale Fig 1 uses *the same figures of the preceding* example of a test The LD_{50} of protection afforded by the vaccine is arrived at in the same manner, i e., subtraction of the two logarithms and derivation of the antilog of the result.

Section 12 *

MODIFIED HABEL TEST FOR POTENCY

For many laboratories producing antirabies vaccine, the lack of relatively large numbers of experimental animals makes the routine use of the standard mouse test for potency impractical. Especially is this true in those laboratories where multiple small volumes of vaccine are prepared at frequent intervals. In these situations a simplified potency test which would screen out vaccines of below-standard antigenic potencies, and which at the same time would be comparable in its minimum requirements to the results obtained by the more complete standard test, would be of value.

In checking results of many potency tests with vaccines of high, low, and intermediate levels of antigenicity, it has been apparent that approximately 500 LD_{50} as an intracerebral challenge dose brings down less than half the immunized mice if the vaccine by the complete test has a potency of over 1,000 LD_{50} protection, which is the minimum requirement.

The technique of the test is the same as the standard procedure (see section 11, page 112) using six intraperitoneal doses of a 0.5% suspension of nervous tissue (0.25 ml), and intracerebral challenge on the 14th day followed by 14 days of observation. Only 20 mice are immunized, and 15 additional mice are held as controls. On the 14th day the standard potency-test challenge virus, diluted to contain 500 LD_{50} per 0.03 ml, is given intracerebrally to the vaccinated mice, and at the same time groups of 5 control mice receive the challenge virus diluted to 10^{-5}, 10^{-6}, and 10^{-7} (see section 11, page 112, for analogous procedure).

To be a valid test, the control titre should indicate that the challenge given to the vaccinated mice was between 100 and 1,000 LD_{50}, and 50% of the vaccinated mice should survive in order that the vaccine pass the screening potency test (for calculation, see section 11, page 112).

It is advised that at periodic intervals a complete standard potency test (see sections 11 and 13, pages 112 and 117) be performed to obtain a more quantitative evaluation of the vaccine over long periods of production in each laboratory

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* Contributed by K. Habel, Chief, Laboratory of Infectious Diseases, National Microbiological Institute, Bethesda, Md., USA

Section 13 *

POTENCY-TEST REQUIREMENTS OF UNITED STATES NATIONAL INSTITUTES OF HEALTH (NIH)

The standard challenge virus (CVS)

The CVS is supplied periodically, usually once a year, to laboratories in the United States of America. This provides a nearly uniform challenge virus and makes possible the evaluation of different lots of vaccines as well as of vaccines from different laboratories. Laboratories are urged to follow closely the procedures outlined. CVS is available to national laboratories upon request to the World Health Organization, Palais des Nations, Geneva, Switzerland.

The working CVS

In the USA, CVS is supplied as a 20% mouse-brain suspension in a 2% horse-serum: distilled-water diluent. This has been stored under dry-ice before shipment; it should be used only if received in the frozen state, and should be retained in this condition until used. The contents of the ampoule should be thawed rapidly under cold running water and then diluted 1 : 4 with the 2% horse-serum diluent. This gives a 5% suspension, which is centrifuged for 15 minutes at a relative centrifugal force of approximately 1,000 g. The supernatant fluid is diluted to 10^{-3} and, using this dilution as the inoculating dose, a sufficient number of normal mice are injected with 0.03 ml intracerebrally to produce the amount of working CVS needed for approximately one year. (One mouse brain will yield approximately 1.5 ml of a 20% suspension.) When an inoculated mouse has shown signs of rabies for a period of 24 hours, the brain is harvested and immediately frozen with dry-ice. The harvested brains are placed in a common container and when the collection is complete they

are thawed, weighed, and ground to pulp, and enough of the 2% horse-serum diluent is added slowly, while grinding, to yield a 20% final suspension. The suspension is given a lot number and without straining or centrifuging it is distributed into ampoules, 2.0-2.5 ml to each ampoule. The ampoules are flame-sealed, and the contents are quick-frozen and stored at dry-ice temperature (approximately −70°C).

Each step in preparing the working CVS must be carried out promptly so as to ensure the survival of the maximum possible amount of virus. Before use as challenge virus, the LD_{50} value of the lot should be determined in six-week-old mice. The lot is satisfactory provided the LD_{50} value occurs between the $10^{-5.0}$ and $10^{-7.0}$ dilutions, inclusive. The maximum variation in the titre obtained from test to test should not exceed one tenfold dilution when the same lot of challenge virus is used. When all the ampoules of a lot have been used, or at the end of a one-year period, a new reference CVS is obtained from the NIH for preparing a new lot of working CVS. This is essential in order to assure uniformity of the working CVS among all laboratories producing rabies vaccine.

Note. The difficulties in shipping frozen material necessitate that WHO send the CVS strain in dried form to laboratories requesting it. A central laboratory can prepare its own primary pool of CVS by one passage of the strain in a sufficient number of mice calculated to meet its needs for several years. If dry-ice facilities are available, the primary pool in flame-sealed ampoules should be kept frozen at −70°C, and the procedure followed as described above. If dry-ice facilities are lacking, the material can be kept frozen at −20°C or lower, the lower the better, but periodic titrations in mice are required to determine possible falls in titre. It is not advisable to work directly from the dried form of CVS because the titre may be too low.

Type of test mouse

The test is based on the use of white Swiss mice approximately four weeks old, uniform in weight (11-15 g), and of one sex.

Reference vaccine

An NIH Rabies Reference Vaccine (ultraviolet-light inactivated and dried) is supplied by the NIH to vaccine-producing laboratories in the USA on request. When the contents of each ampoule are dissolved in 8 ml of distilled water containing 0.25% phenol and 0.01% thiomersal (merthiolate), the vaccine is considered to be a 10% suspension. The reference vaccine is stored at 2°-10°C at all times, whether in the liquid or the dried state. After reconstitution, the vaccine is used not later than two weeks after the diluent is added.

At the request of WHO a limited quantity of NIH Rabies Reference Vaccine 150 C has been made available, and will be supplied by WHO to national laboratories upon demand Since only [illegible] very limited quantity of the dried reference vaccine will be available, it is suggested that national laboratories prepare a sufficient quantity of a dried vaccine of their own to meet their needs for at least one year, and then test their own dried vaccine with the NIH Reference Vaccine. The advantage of using a dried reference vaccine is its stability, and thereby its usefulness for future comparison of vaccines

Immunization of mice

Three or more dilutions of each vaccine under test are prepared in [illegible] vaccine to protect 50% of the mice The range will depend upon the strength of the challenge dose, the kind of mice used, and the potency of the vaccine With a challenge dose of 20 LD_{50} (see page 120) a dilution range of 1 0%-0 2%-0 04% of brain tissue has worked out well for both the NIH Reference Vaccine 150 C, and commercial vaccines produced [illegible] the USA.

At least 16 mice are injected intraperitoneally with 0.5 ml of each dilution of vaccine. Two doses of vaccine are given to each mouse one week apart Enough control mice are set aside at the time the test mice receive the first dose of vaccine for an adequate titration of the challenge virus to be made with at least 10 mice for each dilution of virus (a total of 30-40 control mice).

For vaccination of mice use a 1-ml or 0 5-ml tuberculin syringe and give each mouse 0.50 ml intraperitoneally. For rapid work it [illegible] necessary to etherize the mice Grasp the mouse by the skin of the neck between the thumb and forefinger and clasp the tail between the little finger and palm The mouse is then held firmly on its back and cannot get away even if not etherized The needle best suited for vaccination is the 25-gauge ½- or ¾-inch (0 50-mm × 12- or 18-mm) needle Inoculate in the midline immediately above the umbilicus This avoids the stomach, liver, and bladder Insert the needle, with the bevel up, [illegible] a 45° angle from the head end of the mouse, and inject the vaccine suspension rapidly (see fig 1) If there is any doubt as to whether the needle point is in the peritoneal cavity, move the point from side to side and observe the skin.

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[1] [illegible] ml M/15 KH_2PO_4, [illegible] ml M/15 Na_2HPO_4 $12H_2O$ 900 ml distilled water

FIG. 1. INTRAPERITONEAL INOCULATION OF MOUSE

By courtesy of Dr F. Perez-Gallardo, Madrid

The order of vaccination is not important, so that all the mice to be vaccinated with one lot of vaccine may be kept in one or two boxes.

Challenge of control and test mice

All mice are challenged intracerebrally 14 days after the first dose of vaccine.

One ampoule of the pooled first-passage working CVS is thawed rapidly under cold running water and diluted 1 : 4 with 2% horse-serum : distilled-water diluent. This mixture is then centrifuged for 15 minutes at a relative centrifugal force of approximately 1,000 *g*. The supernatant fluid is a 5 × 10[illegible] dilution of the challenge material, and is used to make further dilutions. All dilutions are made in the same diluent as originally used. It is recommended that the dilutions of the challenge virus be held in an ice-water bath [illegible] equivalent, during the performance of the test in order to pre[illegible] loss.

The imm[illegible] are challenged with a dilution of virus which has been fou[illegible] tests to contain approximately 20 LD_{50} of virus.

Each laboratory, therefore, must determine for itself the dilution of working CVS which will contain the test dose of 20 LD_{50} virus; for example, if the working CVS has been found to have a titre of $10^{-6.5}$ or 1: 3,160,000, then the 20 LD_{50} would be contained in a dilution of 1: 158,000 or $10^{-5.2}$. This dilution is prepared from the 5×10^{-2} supernatant described above, as follows:

1 ml (of 1: 20) + 4 ml diluent = 1 : 100
1 ml (of 1: 100) + 9 ml diluent = 1 : 1,000
1 ml (of 1 : 1,000) + 9 ml diluent = 1 : 10,000
1 ml (of 1 : 10,000) + 14.8 ml diluent = 1 : 158,000

After all immunized mice have been inoculated with the test dose of virus (0.03 ml of the 1 : 158,000 dilution in the example given above), one group of control mice is inoculated with the same test dose of virus. Two tenfold dilutions of the test dose are then prepared for injection of the remaining control mice. It is desirable to inject the 1 : 100 dilution and the 1 : 10 dilution of the test dose of virus in that order. In this way one syringe may be used to challenge all the immunized mice and one group of control mice, another syringe must be used to challenge the remaining control mice.

All mice are observed for 14 days from the time of the challenge injection. Only those deaths occurring after the fifth day and those preceded by signs of fixed-virus rabies (paralysis, convulsions) are considered deaths from rabies. Any mice becoming paralysed but surviving the 14-day observation period are considered as equivalent to deaths from rabies.

The definitions of "paralysis" and "convulsions", as applied to mice following the injection of the challenge virus, are as follows:

Paralysis is the partial or complete loss of motor power of one or more legs.

Convulsions are indicated by violent and abnormal muscular contractions of the body—often termed spasms. These are brought about by external stimulation, such as a disturbing sound, or handling.

For the test to be considered valid, the results obtained after challenge of the immunized mice must show that the dilutions of the reference vaccine encompass the 50% end-point—that is, the majority of the mice receiving the highest dose of vaccine survive, and the majority of the mice receiving the smallest dose of vaccine die. For the test vaccine, unless an exact end-point is especially desired, it is only necessary that the majority of the mice receiving the highest dose of vaccine survive. The virus titre of the test dose of challenge virus is not particularly important, provided that it falls between 5 and 50 LD_{50}. It is necessary, however, that all the control mice receiving the test dose of virus die. Because of the nature of the virus

and the test involved, it is difficult to obtain exactly the same virus titre from one test to another.

Potency test on live or attenuated virus vaccine

Vaccines containing live or attenuated virus, except for chicken-embryo vaccines (see section 15, page 125), can be tested in the same manner as killed-virus vaccines.

Calculation of potency (antigenic value)

50% end-points are determined for both the reference vaccine and the test vaccine by the method of Reed & Muench. The end-point is calculated as a 50% effective dose (ED_{50}) of vaccine in milligrams of original brain tissue which will protect 50% of the mice. An abstract of this method is given in Annex 1 below. By dividing the ED_{50} of the reference vaccine by the ED_{50} of the test vaccine, a value is obtained which is the antigenic value of the test vaccine in terms of the reference vaccine. The LD_{50} of challenge virus received by the immunized mice is calculated by dividing the dilution of virus used as the test dose by the 50% end-point dilution of virus in control mice, as calculated by the method of Reed & Muench.

Antigenic value requirement (see page 123 for computation)

The antigenic value of a vaccine under test should be 0.6 or higher in relation to NIH Reference Vaccine 150 C. For each new lot of reference vaccine, an antigenic value will be determined in terms of the previous reference vaccine. It is anticipated that there will be minor differences in potency of future batches of reference vaccines in spite of efforts to keep these variations to a minimum.

Annex 1

METHOD OF DETERMINING 50% END-POINTS BY THE METHOD OF REED & MUENCH[2] AS APPLIED TO THE POTENCY TEST OF RABIES VACCINE IN MICE

The 50% effective dose (ED_{50}) is calculated as the amount of brain tissue which [illegible] 50% of the mice against a subsequent challenge with rabies [illegible]s of the challenge are arranged in columns

[2] Reed, L. J. [illegible] Amer. J. Hyg. 27, 493

indicating the number of mice which survived and which died on each dose of vaccine. The value for survived and dead in the columns under the heading *Reconstructed totals* are then obtained by adding the survivals from the high to the low dilutions, and the deaths in the opposite direction.

EXAMPLE

Dilutions of 5% brain tissue vaccine	Brain tissue (mg)	Number of mice	Survival	Dead	Reconstructed totals survived	Reconstructed totals dead	Percentage mortality
1 5	10	16	10	[illegible]	↑ 19	↓ [illegible]	6/25 = 24
1 25	2	16	8	8	9	14	14/23 = 61
1 125	0 4	16	1	15	1	29	

From this tabulation, the 50% end-point dilution lies between 2 mg and 10 mg of brain tissue, and is obtained by the following calculation:

A

$$\frac{61\ (\text{mortality next over } 50\%) - 50}{61\ (\text{mortality next over } 50\%) - 24\ (\text{mortality next under } 50\%)}$$

× the logarithm of the dilution factor (in this example dilution factor is 5)

= logarithm of dilution factor at 50% end-point

or $\frac{11}{37} \times 0\ 699 = 0\ 208$ (log of dilution factor at 50% end-point)

B Antilog of dilution factor at 50% end-point × amount of brain tissue injected into mice at dilution at which more than 50% of mice died = ED_{50}

since antilog 0 208 = 1 61
then 1 61 × 2 0 mg = 3 22 mg = ED_{50}

The antigenic value of a test vaccine is obtained by dividing the ED_{50} of the test vaccine into the ED_{50} of the reference vaccine, e g,

reference vaccine ED_{50} = 3 22 mg BT (brain tissue)
test vaccine No 1, ED_{50} = 1 07 mg BT

antigenic value of vaccine No $1 = \frac{3\ 22}{1\ 07} = 3\ 0$

The challenge dose of virus is calculated from the results of challenge of the control mice. The results of the challenge are arranged in columns and a *Reconstructed totals* column is obtained in a manner similar to that described above

EXAMPLE

Dilution of virus	*Number of mice*	*Survived*	*Dead*	*Reconstructed totals survived*	*Reconstructed totals dead*	*Percentage mortality*
$10^{-5.2}$	10	0	10	↓ 0	↑ 18	
$10^{-6.2}$	10	4	6	4	8	8/12 = 67
$10^{-7.2}$	10	8	2	12	2	2/14 = 14

Since the 50% end-point dilution or LD_{50} lies between $10^{-6.2}$ and $10^{-7.2}$ the logarithm of the LD_{50} dose is found as follows:

$$\text{Log of } LD_{50} \text{ dose} = 6.2 + \frac{67-50}{67-14}$$
$$= 6.2 + \frac{17}{53}$$
$$= 6.2 + 0.32$$
$$= 6.52$$

The LD_{50} dose, therefore, is $10^{-6.52}$, or the undiluted virus suspension contained 3,310,000 LD_{50}. Since a $10^{-5.2}$ dilution of virus was used as the challenge dose, that dose contained:

$$\frac{3,310,000}{158,000} \text{ or } 21\ LD_{50}.$$

challenged similarly with a 1 : 5,000 dilution (3 LD_{50}). All vaccinated animals should survive and the controls should die.

Note: The test technique described above was devised for use with the Louis Pasteur strain of fixed-virus rabies at its present stage of adaptation to the rabbit at the Institut Pasteur, Paris (1,901st passage 1 January 1953)

When another strain of fixed-virus rabies adapted to the rabbit is used, it is as well to perform a preliminary titration of the virus in the rabbit by the intracerebral route, determining the dilutions of virus on either side of the LD_{50}

For intracerebral test on the 30th day in rabbits which have been vaccinated for 10 days, 1.5 LD_{50} should be used, in rabbits which have been vaccinated for 20 days, the concentration should be 3 LD_{50}.

It would appear unnecessary to interpret these results mathematically by the cumulative method of Reed & Muench, the minimum lethal dose being easy to determine and not varying under the experimental conditions.

However, it is clear that this virulence titre varies not only according to the strain of the fixed virus selected for the test, but also according to the number of passages undergone by the strain, the weight and characteristics of the domestic rabbits used, and the climate where the test is carried out. Nevertheless, there is no reason why these factors should alter the minimum lethal dose provided this has been determined under the same experimental conditions (see Note on page 127).

The results of the test are available in 8 days, but it is advisable to keep the animals for a month after the challenge inoculation to make certain of the results, since in exceptional cases (2% of cases) rabies can develop with some delay.

Vaccination

Once the virulence titre is known, it is possible to evaluate the protective power of the rabies vaccines by testing batches of five rabbits on the 30th day of vaccination with increasing dilutions of fixed virus

Thus the Paris Institut Pasteur phenolized vaccine (see section 6 (A), page 77), on being injected subcutaneously into the flank of the animal at the rate of 2 ml per day during 20 consecutive days, protects a 2 kg rabbit inoculated intracerebrally on the 30th day of vaccination against a 1 : 5,000 virus dilution (3 LD$_{50}$).

If the volume of the vaccinal injections (i.e., the quantity of vaccine inoculated and the duration of vaccination) is varied, the results obtained show that massive injections are more effective than repeated ones. When 2 ml are injected daily for 10 consecutive days, phenolized vaccine protects a 2-kg rabbit intracerebrally inoculated on the 30th day against a 1 : 10,000 virus dilution (1.5 LD$_{50}$). However, when 1 ml is injected daily for 20 consecutive days, it does not protect the rabbit against a 1 : 16,000 dilution, thus tending to confirm the importance of early preventive treatment and massive and early injections in man after a suspected bite.

For an actual test a minimum of 6 rabbits is used, although it is preferable to use 12 animals, divided into groups of equal number. Group 1 receives 2 ml of vaccine (5% tissue suspension) for 10 days. Group 2 receives 2 ml of vaccine (5% tissue suspension) for 20 days. Group 3 is used as control. Thirty days after commencing the administration of vaccine, Groups 1 and 3 are challenged intracerebrally with 0.25 ml of a 1 : 10,000 dilution (1.5 LD$_{50}$) of Pasteur-strain fixed virus; Group 2 is

FIG. 1. INOCULATION INTO MASSETER MUSCLE, USING DOG

By courtesy of Dr F Perez-Gallardo Madrid

28-35 days old These mice are observed for 21 days and the number of dead mice is recorded daily. After the period of observation, the LD_{50} titre of each preparation is calculated (for calculation see section 13, page 122)

In order to make a large virus pool, only those glands of which the titre exceeds $10^{-4.5}$ LD_{50} are pooled.

Preparation of the pool

The glands are removed from the freezer, thawed, cut into small pieces with scissors, and put into a Waring blendor with enough sterile 10% normal rabbit serum physiological-salt-solution to make a 20% suspension by weight The blendor is set in motion for 2 or 3 minutes only, in order

Section 15 *

POTENCY TEST FOR CHICKEN-EMBRYO VACCINE

The test consists of the intramuscular inoculation of guinea-pigs with chicken-embryo vaccine, followed three weeks later by challenge of the animals with street virus.

Guinea-pigs

Guinea-pigs weighing not less than 400 g should be chosen. The use of lighter animals is inadvisable.

Immunization procedure

If the presently available chicken-embryo vaccine (33% tissue suspension) is used, 0.25 ml of 5% tissue suspension per guinea-pig should be injected intramuscularly into the gastrocnemius muscle of the right hind leg of each guinea-pig. The vaccine should be rehydrated with sterile distilled water shortly before inoculation and the required dilution should be made in sterile, distilled water.

Preparation of challenge material

Adult dogs are injected with 0.1 ml of a suspension of infected canine submaxillary-gland tissue kept frozen at −70°C. The injection is bilateral into the masseter muscle, using a 1-ml syringe and a 20-gauge, 1-inch (0.90 × 25-mm) needle (see fig. 1). When the animals die after showing signs of rabies, or when they are sacrificed when moribund, the submaxillary-gland tissue is removed, a small piece is separated by cutting with scissors, and the remainder is frozen in a large Petri dish and kept in a refrigerator at −50°C to −70°C. The selected portion is ground in a mortar with enough 10% normal rabbit serum : physiological-salt-solution to make a 10% suspension by weight. This suspension is then centrifuged in an angle-head centrifuge for one minute at 1,000 revolutions per minute (r.p.m.) and the supernatant liquid is separated. Tenfold dilutions of the supernatant liquid are made in 10% normal rabbit serum : physiological-salt-solution and 0.03 ml is injected intracerebrally into Swiss albino mice

* Contributed by Hilary Koprowski, Assistant Director, Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N.Y., USA

TABLE I. RESULTS OF POTENCY TEST FOR CHICKEN EMBRYO VACCINE

Batch of vaccine	LD_{50} titre in mice	Results of intramuscular challenge with street virus								
		guinea-pigs							dogs	
		mortality ratio of challenge animals							mortality ratio	
		immunized with dilutions of vaccine						non-vaccinated controls	vaccinated **	non-vaccinated controls
		1 5	1 20	1 80	1 320	1 1,280	1 5,120			
A *	$10^{-2.75}$	0 8	0 9	0 8	0/10	0 8	—	10,10	0 5	3,6
B	$10^{-2.85}$	0 9	0/10	5/10	5/10	9 10	—	10/10	0 6 } 0 3 } two tests performed	3 5 10 10
C	$10^{-2.95}$	0/8	0 8	2/7	8 8	—	—	10/10	0 9 } 0 6 } two tests performed	8 9 6/10
D *	$10^{-3.00}$	0 8	0,6	1/10	4,5	0/9	—	8 8	0 10	10,10
E	$10^{-3.20}$	—	—	1/4	1/6	4/5	0 4	8 9	0,6	18 25
F	$10^{-3.00}$	—	—	0 5	0/3	1/4	1 5	8 9	0 8	18/25
G	$10^{-3.00}$	—	3 15	—	—	—	—	13/16	0/7	3 5

* 1 ml of vaccine containing 5% tissue suspension was injected into each guinea-pig ; in all remaining instances 0 5 ml was used.
(Note 0 25 ml of 5% tissue suspension is now used for routine testing)

** 5 ml of 20% tissue suspension were used for vaccination

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[illegible] pools have been used for challe[illegible] [illegible] it has been observed that 0 1 ml of [illegible] [illegible] of canine salivary-gland [illegible] [illegible] brain and prepared as outlined [illegible] [illegible] challenge inoculum

[illegible] of the control animals should die of rabies [illegible] [illegible] should survive challenge [illegible] [illegible] of rabies This test very closely [illegible] [illegible] and can be performed with relatively [illegible] [illegible] with vaccine and one challenge [illegible] [illegible] locally isolated street-virus strain for challe[illegible] [illegible] used to evaluate the potency of [illegible] [illegible] geographical areas of the world against [illegible] [illegible] It has a disadvantage in that [illegible] challenge virus However [illegible] [illegible] employed, a relatively small [illegible] [illegible] which can be used for [illegible]

Part IV

PRODUCTION OF HYPERIMMUNE SERUM

animals. So far, only two street-virus pools have been used for challenge purposes in this laboratory, and it has been observed that 0.1 ml of 1:40 dilution or 0.2 ml of 1:80 dilution of canine salivary-gland tissues, infected with the New York City strain and prepared as outlined on page 129, constituted an excellent challenge inoculum.

In the actual test, 80% of the control animals should die of rabies and 70% of the vaccinated guinea-pigs should survive challenge inoculation without showing any symptoms of rabies. This test very closely parallels events occurring in nature and can be performed with relatively little labour. Only one inoculation with vaccine and one challenge inoculation are required. By employing locally isolated street-virus strain for challenge purposes, the test can be used to evaluate the potency of chicken-embryo vaccines in different geographical areas of the world against different strains of rabies. It has a disadvantage in that it requires dogs for the preparation of challenge virus. However, if a potent preparation of salivary-gland tissue is employed, a relatively small number of dogs will suffice to yield a preparation which can be used for a long period of time for challenge purposes.

Part IV

PRODUCTION OF HYPERIMMUNE SERUM

Section 16 *

METHOD USED AT INSTITUT PASTEUR, PARIS

The Institut Pasteur antirabies serum is prepared from horses hyperimmunized with fixed virus. This serum can be produced either in the crude state, or purified and concentrated

Rabies strain

The rabies strain used is the Institut Pasteur fixed-virus strain which has always been employed there for immunization against rabies. The characteristics of the strain and the method of maintaining it in the rabbit are dealt with in section 6 (A), page 77

Method of immunization

The horses are immunized by subcutaneous inoculation. They are first given an initial series of injections of phenolized rabies virus, i e., antirabies vaccine prepared in the same way as that for human use, as described in section 6 (A), page 81, but employing rabbit brain instead of sheep brain The vaccine is used for the first injections after incubation for eight days at +4°C Thereafter, more freshly prepared vaccines are used.

After this initial immunization with phenolized vaccine, the horses next receive the non-attenuated fixed virus Finally, according to the titre reached during immunization, the injections are continued with the alternative addition of adjuvants

Once a suitable titre has been reached, the horses are re-inoculated every month and are then bled again

Immunization schedule and doses

Young horses in a good state of health are used. Immunization is carried out according to the following plan

	First series	*phenolized fixed virus*
First injection	40 ml	5% rabbit brain, phenolized
One week afterwards	[illegible] ml	5% rabbit brain, phenolized
One week afterwards	120 ml	5% rabbit brain, phenolized

* Contributed by Pierre Lépine (Chief, Virus Section) and P Atanasiu, Institut Pasteur, Paris, France

Section 16 *

METHOD USED AT INSTITUT PASTEUR, PARIS

The Institut Pasteur antirabies serum is prepared from horses hyper-immunized with fixed virus. This serum can be produced either in the crude state, or purified and concentrated.

Rabies strain

The rabies strain used is the Institut Pasteur fixed-virus strain which has always been employed there for immunization against rabies. The characteristics of the strain and the method of maintaining it in the rabbit are dealt with in section 6 (A), page 77.

Method of immunization

The horses are immunized by subcutaneous inoculation. They are first given an initial series of injections of phenolized rabies virus, i.e., antirabies vaccine prepared in the same way as that for human use, as described in section 6 (A), page 81, but employing rabbit brain instead of sheep brain. The vaccine is used for the first injections after incubation for eight days at +4°C. Thereafter, more freshly prepared vaccines are used.

After this initial immunization with phenolized vaccine, the horses next receive the non-attenuated fixed virus. Finally, according to the titre reached during immunization, the injections are continued with the alternative addition of adjuvants.

Once a suitable titre has been reached, the horses are re-inoculated every month and are then bled again.

Immunization schedule and doses

Young horses in a good state of health are used. Immunization is carried out according to the following plan:

	First series phenolized fixed virus	
First injection	40 ml	5% rabbit brain, phenolized
One week afterwards	80 ml	
One week afterwards	120 ml	

* Contributed by Pierre Lépine (Chief, Virus Section) and P. Atanasiu, Institut Pasteur, Paris, France.

For this series, as for those following, the injections are made at several points so as to distribute the injection mass and to ensure rapid absorption. Little if any reaction is observed at the site of the injections.

Second series: fresh fixed virus

The second series commences immediately after an interval of one week.

First injection: half a rabbit brain (fresh fixed virus) suspended in 50 ml of distilled water

Second injection: one whole rabbit brain in 100 ml of distilled water

Third injection: two whole rabbit brains in 150 ml of distilled water

A control bleeding is carried out a week after the third injection of the second series. According to the results and the titre obtained, immunization is stopped, or the second series is repeated, adding supplementary antigen injected intramuscularly with adjuvants. Various adjuvants may be employed, but the best results are obtained with sodium alum in a concentration of 4:1,000 in the suspension injected. Mineral-oil derivatives with an emulsifier totalling one-third the quantity of injected material can also be used, but they are less advisable than sodium alum. The final titre of the antirabies serum is normally reached between the second and third month (see section 18, page 139, for potency test).

Maintenance of immunization

Blood is harvested at this stage and the horses are allowed to rest for a month. Subsequently, immunized horses receive every month two rabbit brains (fresh fixed rabies virus), injected as with the third injection of the second series. The horses are bled a week after this reinforcing injection. They are allowed to rest completely for one month during the year.

Section 17 *

METHOD USED AT ISTITUTO SIEROTERAPICO E VACCINOGENO TOSCANO, SIENA

Of the various animal species, whether small or large in size, the equine —in particular the horse—should be considered the animal of choice for the preparation of antirabies serum.

The production of hyperimmune serum in the horse, started by Fermi in 1909, is related to the quality of the antigen used, the doses injected, and the rhythm of the injections. The antigen used by the authors consists of Fermi's phenolized antirabies vaccine ("Sassari" virus), freshly prepared, and injected subcutaneously in a dose of 10-20 ml every day for a month

A bleeding 7-8 days after the last injection of the first series shows, however, that the presence of virucidal antibodies in the animals' sera is still not high at this stage, although they are clearly present in all the subjects treated. On giving the animals a rest period of a month and carrying out a second series of injections, a serum whose virucidal value is double, treble, or even higher is obtained After a further rest period of from two to three months, and following another series of vaccine injections, a very powerful serum is obtained, of which a 1-ml dose is capable of neutralizing up to one million or more doses lethal for the guinea-pig

The production of a good antirabies serum in a fresh horse appears, however, to be very slow, calling for several rest periods alternating with an immunization series On the other hand, antibodies are rapidly and intensively produced in horses which have undergone previous immunization

Animals which have received a basic immunization with phenolized vaccine are also not harmfully affected by the injection of high doses of living and virulent virus, thus making it possible to bring about immunization more readily As in the production of antitoxic sera (diphtheria and tetanus), animals which are good producers of serum are encountered side by side with mediocre or even refractory subjects. On a reduced scale, the course of antirabies serum production is very similar to that of antitetanus serum, the rapidity and intensity of production of this is well

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* Contributed by D d Antona & E Falchetti Istituto Sieroterapico e Vaccinogeno Toscano, Siena, Italy

known in the case of horses subjected to repeated cycles of immunization, or to previous vaccination. It is thus possible to prepare a powerful mixed antitetanus and antirabies serum at the same time by simultaneous injection into the animal of the corresponding antigens (tetanus toxoid or toxin, and antirabies vaccine). As with the tetanus antitoxin, the virucidal antibodies seem to be distributed in the pseudoglobulin fractions of the serum, and to a reduced extent in the euglobulin fraction. The antirabies serum may be concentrated and purified, however, by the same techniques as apply to the antitoxic sera, including peptic digestion.

The antirabies serum is remarkably stable and retains its properties in a refrigerator for several years, as well as resisting even prolonged heating at 58°-60°C.

Titration of the virucidal antibodies is carried out by the authors using in vitro mixtures of different dilutions of the serum and an emulsion of virus (brain tissue) with a definite, known virulence. After standing for one hour at 37°C, the mixtures are injected intracerebrally into guinea-pigs in a dose of 0.2 ml, and the titre is calculated on the basis of the dilutions able to protect 80% of the animals.

POTENCY TEST OF ANTIRABIES SERUM

Potency test

This test is essentially the same as the serum-virus neutralization test in mice described in section 5, page 69 The test must be performed with serum before the addition of any chemical preservatives

Test animal: Normal mice of either sex weighing 10-14 g each are used In any one test, mice of only one sex are used.

Test virus: Any standard strain of rabies virus of known potency may be used

Reference antirabies serum: A reference serum distributed by WHO will be available for use in the potency test for comparative purposes when standardizing all antirabies sera This serum will be supplied in the dry form, and instructions for redissolving will be sent with each shipment. Its potency is adjusted so that when mixed in final concentration with not less than 31 6 LD_{50} and not more than 316 LD_{50} of one part of virus suspension, the minimum protective titre (50% end-point) will be not less than 1 300 dilution.

Procedure: Serial twofold dilutions of both the serum under test and the reference serum are prepared in 2% normal serum in distilled water or physiological salt solution Six serial twofold dilutions, starting at 1 : 50 and continuing through to 1 : 1,600, are usually sufficient for the reference serum, and 1 125 through to 1 4,000 for the serum under test These dilutions will reveal a 2 5 potency factor required of the serum under test Equal quantities of a suspension of test virus are added to the dilutions of serum. The mixtures are incubated in a 37°C water-bath for one hour, and 0 03-ml quantities are injected intracerebrally into the mice. At least 10 mice are injected for each mixture The amount of virus used is such that each mouse receives not less than 31 6 LD_{50} and not more than 316 LD_{50}. The mice are observed for two weeks For a serum under test, its minimum acceptable potency should be 2.5 times that of the reference serum

* Contributed by Hilary Koprowski Director Viral and Rickettsial Research Lederle Laboratories Division American Cyanamid Company Pearl River N Y , USA

Sterility test

The contents of the final container should be sterile, as indicated by culturing the entire recommended dose, except that the amount cultured need not exceed 5.0 ml. The culture should be made in one or more tubes of fluid thioglycollate medium or other standard media with sufficient dilution for the preservative no longer to exert a bacteriostatic effect. Incubation is at 32°C, with observation for at least 7 days.

Part V

LABORATORY ANIMALS

Section 19 *

BREEDING AND CARE OF LABORATORY ANIMALS

Breeding stock

This is perhaps the most important matter in the establishment of a breeding colony for mice or other laboratory animals Every variety of animal has certain natural diseases, many of which can be eliminated by proper segregation of mature animals and testing for the presence of disease in isolated families.

The inbred white mouse is the most widely-used experimental animal in virus laboratories. Certain genetic lines of white mice have been found to be highly susceptible to a wide variety of virus diseases Other genetic lines or varieties of white mice are known to be resistant to certain virus diseases; for instance, one genetic variety of white mice is resistant to 17 D yellow-fever-vaccine virus, and when inoculated intracerebrally with this virus the mice do not sicken and die, while other genetic types uniformly die of this infection

Breeding stock can be obtained from mouse colonies that have been tested and found free of paratyphoid and salmonella intestinal bacterial infections, bartonella and toxoplasma parasites, and the more common viruses such as mouse encephalomyelitis, lymphocytic choriomeningitis, and ectromelia

It is possible that vaccinia virus used for immunization of man may be introduced into a mouse colony and may produce a disease similar to, and antigenically identical with, ectromelia disease There is a mite-borne rickettsial infection of wild mice caused by *Rickettsia akari*, which could be introduced into breeding colonies of mice Faecal droppings of wild mice can introduce mouse encephalomyelitis virus into breeding boxes and so infect breeding stock previously known to be free of this disease

All lines of white mice appear to be uniformly susceptible to rabies street virus inoculated intracerebrally

Housing

The colony of normal mice should be in a room by itself and inoculated mice must not be allowed in the same unit It is preferable to have

* Contributed by Harald N Johnson Virus Research Centre Poona, India

the breeding colony on another floor or in a different building from the room used for infected animals.

The windows of the breeding room must be screened *to keep out flies* and mosquitos, as these insects may introduce bacterial, *parasitic, and* virus infections. The entry to the breeding room should have a vestibule *with a screened door.* The floor should be washable and of a material which would prevent entry of wild mice or other rodents. The roof should be constructed or reconditioned to prevent the entry of rodents and birds.

Mice cannot stand high temperatures, and the temperature of the breeding room must be kept at under 100°C.

Caretakers for animals

Persons who work in the rooms containing infected mice should not be allowed to do any of the work in the breeding quarters for normal mice. Caretakers should be instructed to scrub the hands with a brush in soap and water, and to clean the finger-nails with a stick before beginning work and whenever there is a likelihood of contamination. Intestinal infections of mice, such as paratyphoid and salmonella, may be introduced by caretakers' handling food or water-bottles with contaminated hands

Cages and bedding

Cages for mice are ordinarily constructed from galvanized iron with a heavy wire-mesh section in the lid for ventilation. Water-bottles are necessary for breeding boxes. [illegible] of the [illegible] ishing [illegible] Wood [illegible] shells may be used for bedding.

Food

All food must be protected from rodents and insects by storage in metal containers with suitable lids. Whole grain such as unmilled wheat is a complete food for mice. For breeding stock it is advisable to include powdered milk in the diet An excellent method of feeding mice is to prepare a whole-wheat cereal containing reconstituted powdered milk and salt, allow it to set, and then cut it into blocks for feeding This furnishes both food [illegible] d water

Method of [illegible] ng up breeding colony

For rapid [illegible] reeding it is best to keep two females and one male together all the time [illegible] the breeders are discarded. This ensures a new litter

every three weeks and simplifies the handling of the animals. For some purposes it may be preferable to have five females and one male in a stock breeding box and to remove the females when they become obviously pregnant; these are then put into a box by themselves and returned to the same breeding box at the end of the weaning period This method gives a check of the number of mice per litter and a more exact record of age, but it is not justified in most diagnostic laboratories as it takes too long to produce a litter from each mouse. Breeder females which do not become pregnant in three weeks are removed from the breeding colony and used for routine purposes.

Testing for presence of disease in breeding colony

It is possible to test for the presence of mouse encephalomyelitis virus by collecting a stool-specimen pool from a box of 50 mice of weaning age. This material should be ground in a mortar with saline solution to make a concentration of about 10% and centrifuged at top speed in an ordinary horizontal or angle-head centrifuge for one hour to sediment as much of the bacterial content as possible. Then take off the supernatant fluid, add to it penicillin to a concentration of 500 units per ml and streptomycin to a concentration of 1 mg per ml, and test this material by inoculation of 0 03-ml amounts intracerebrally into each of 10 mice Use six-week-old mice for the test. If paralysis develops in the inoculated mice at from 4 to 30 days after inoculation, check brain impressions for bacteria by staining and culture methods; if no bacteria are found in the stained preparations, subpass to other mice and, if these are consistently infected, check specificity of the virus by serum neutralization test If encephalomyelitis virus is present in the colony there should be no difficulty in obtaining the virus by the method outlined above Most laboratory colonies of white mice harbour this virus in the intestinal tract and only rarely do mice develop paralysis The virus may be isolated in subpassage studies by chance selection of a mouse having a systemic infection with the natural virus There are instances where subpassage in mice of a neurotropic virus, such as rabies fixed virus, may result in loss of the fixed virus and the maintenance of a fixed strain of mouse encephalomyelitis virus of about the same incubation period as that produced by fixed rabies virus. Subpassage of the virus through rabbits will eliminate the mouse encephalomyelitis virus infection. Hamsters are susceptible to infection with the mouse encephalomyelitis virus when inoculated intracerebrally

For lymphocytic choriomeningitis virus and several other chronic infections of mice, it is best to test individual spleen specimens from at least 10 mice selected at random from among two- to three-week-old mice obtained from the breeding colony Prepare suspensions of spleen tissue

by grinding in a mortar in saline solution to make a 10% tissue suspension. Centrifuge at low speed for 10 minutes, or preferably allow to settle by standing in the refrigerator for 30 minutes; then take off the supernatant fluid and test by intracerebral inoculation of 0.03 ml into each of 10 mice. Toxoplasma infection may also be picked up by this method, although if this is suspected it is best to test the whole tissue suspension without centrifugation. The presence of toxoplasma in brain impressions can be determined by fixation of the impressions in methyl alcohol and staining with Giemsa stain. The course of the disease and incubation period of toxoplasma infection and rabies fixed-virus infection is similar in mice infected by intracerebral inoculation. Information on other diseases will be found in the UFAW handbook edited by Worden, for which the reference is given on page 149.

Ectoparasites of mice

Mice should be examined for the presence of ectoparasites such as mites and mange parasites. If found, such conditions may be handled by dipping the mice or treating them with insecticides. Natural infections of mite origin must be prevented by not allowing wild mice access to the mouse breeding quarters.

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ADDITIONAL REFERENCE SOURCES

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No. 67

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Second report of the Joint FAO/WHO Expert Committee
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No 40

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Report on the first session of the Joint WHO/FAO Expert Group
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34 pages, second impression, 1953

The [illegible] considers the twofold aspect of brucellosis as a world problem—*(a)* in public he[illegible]h as a disease transmitted from animals to man, and as a threat to food prod[illegible]d *(b)* in animal industry, since it produces abortion, decreased milk yield, and t[illegible] *or permanent infertility among livestock*. Detailed recommendations for huma[illegible]ns and for control of the disease in cattle, sheep, and goats are given.

In annexes to the panel's report, the control of brucellosis in swine is discussed, and various tests and [illegible]sed in the bacteriological diagnosis of the disease are described.

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